Long-Term Deprivation of Substance P in PPT-A Mutant Mice Alters the Anoxic Response of the Isolated Respiratory Network

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Telgkamp, Petra, Yuqing Q. Cao, Allan I. Basbaum, and Jan-Marino Ramirez. Long-term deprivation of substance P in PPT-A mutant mice alters the anoxic response of the isolated respiratory network. J Neurophysiol 88: 206–213, 2002; 10.1152/jn.00676.2001. The aim of this study was to elucidate the role of the neuromodulator substance P and its related tachykinin neurokinin A (NKA) in normoxic and anoxic conditions using medullary slice preparations. The effect of a blockade of endogenous substance P was assessed by an acute pharmacological blockade of the receptors with spantide in wild-type animals and by the use of preprotachykinin-A (PPT-A) mutants. These mutants lack from birth the PPT-A gene, which codes for the precursor of substance P and NKA. Spantide treatment reduced frequency (~37%, n = 9) and regularity (twofold) of eupneic-like respiratory activity under normoxic conditions, whereas in PPT-A mutants, eupneic-like activity was under normoxic conditions not significantly different from the wild-type mice (WT). The response to short anoxic episodes (5 min) was characterized in the WT by an increase in respiratory frequencies at the onset of anoxia (ratio anoxic/control frequency = 1.9 ± 0.2, n = 18). This anoxic ratio was unaltered in the presence of spantide (ratio = 2.3 ± 0.4, n = 8) but increased in the mutant (ratio = 4.1, n = 15). We conclude that endogenously released substance P is important for the maintenance of regular respiratory activity. Short-term blockade of substance P receptors decreases the frequency and regularity of rhythmic activity. Long-term deficiency in substance P leads to compensatory mechanisms that result in an apparently normal respiratory activity under normoxic conditions but a significantly altered response of the respiratory network during anoxia.

INTRODUCTION

Rhythmic motor activity has to adapt to changes in behavioral and environmental conditions. Neuromodulators, such as amines and peptides, play a key role in this adaptation process. Released in a state-dependent manner, they can change the network’s activity by altering membrane and synaptic properties of rhythm-generating neurons. In invertebrate neural networks, it has been demonstrated that neuromodulators continually orchestrate the configuration of a neural network. Thus coordinated network activity depends on the presence of a fine-balanced blend of different neuromodulators (Ayali and Harris-Warrick 1999; Blitz et al. 1999; Marder 2000). Short-term changes in this modulatory milieu can transform the network into a different state. Interestingly the response to neuromodulators is not static. There is increasing evidence that dependency on the presence of certain neuromodulators can drastically change after long-term depletion of a neuromodulator (Golowasch et al. 1999; Thoby-Brisson and Simmers 1998, 2000). The capacity of a neural network to adapt to the absence of a neuromodulator is of great medical interest as many disease states are characterized by long-term deficiencies in certain neuromodulators.

Here, we characterized the effect of short- and long-term manipulation of the neuromodulator substance P on the mammalian respiratory network. The respiratory network is located within the lower brain stem in the so-called pre-Bötzinger complex (Ramirez et al. 1998b; Smith et al. 1991), and it can be isolated in medullary transverse slice preparations of mice. This slice preparation generates under normoxic conditions two types of fictive respiratory patterns, eupneic respiratory activity and sighs (Lieske et al. 2000; Ramirez et al. 1998a; Telgkamp and Ramirez 1999). The generation of these activity patterns is state dependent. In response to hypoxic conditions, there is an initial increase in the frequency of eupneic and sigh activity (augmentation), which is followed by a secondary depression of respiratory activity, and apnea (Lieske et al. 2000). Such a bi-phasic response resembles qualitatively the hypoxic response of the intact in vivo respiratory network (Bureau et al. 1984; Lawson and Long 1983; Neubauer et al. 1990). In the intact network, additional inputs from peripheral chemoreceptors and higher brain centers (e.g., the pons) modulate this hypoxic response. Several peptides and amines are known to play an important role in modulating respiratory activity during hypoxia (Greer et al. 1995; Neubauer et al. 1987; for review see Bianchi et al. 1995; Bonham et al. 1995). A particularly important peptide is substance P (SP). It acts not only on respiratory-related neurons located in the peripheral nervous system, specifically within the carotid body (Cragg et
al. 1994; Prabhakar et al. 1989, 1990), but also within the CNS (Gillis et al. 1980; Menetrey and Basbaum 1987). Released in the brain stem (Arregui et al. 1981; Bonham 1995; Lindefors et al. 1986; Srinivasan et al. 1991), SP mediates the hypoxic drive from the peripheral chemoreceptors (De Sanctis et al. 1991; Gillis et al. 1980; Kumar et al. 2000a,b; Prabhakar et al. 1987, 1993, 1995; Yamamoto and Lagerranz 1985) and acts also directly on the respiratory rhythm-generating network. SP antagonists cause hyperventilation in vivo (Chen et al. 1990a,b) and SP agonists increase the respiratory frequency in vitro (Johnson et al. 1996; Ptak and Hilaire 1999; Ptak et al. 1999). Anatomical evidence indicates that SP immunoreactive fibers project directly into the ventral respiratory group (VRG) (Holtman and Speck 1994) and that the neuropeptide I (NK1) receptor, a subtype of the SP receptor, is specifically expressed within the pre-Bötzinger complex (PBC) (Gray et al. 1999; Liu et al. 2001; Wang et al. 2001). About one-third of preinspiratory neurons in the PBC are positive for NK1 receptors (Guyenet and Wang 2001). Furthermore, a specific and near complete bilateral destruction of NK1-sensitive PBC neurons results in both an ataxic breathing pattern with markedly altered blood gases and pH and pathological responses to challenges such as hyperoxia, hypoxia and anesthesia (Gray et al. 2001).

The aim of this study was to elucidate the role of the neuromodulator substance P and its related tachykinin neurokinin A (NKA) in the homeostasis of respiratory activity. Preprotachykinin-A (PPT-A) mutants were used as a genetic model for studying the long-term depletion of this important neuromodulator as, from birth, they lack the PPT-A gene, which codes for the precursor of SP and NKA (Cao et al. 1998). These results were compared with the effect of an acute short-term pharmacological manipulations suggest that the respiratory network is capable of compensating for the loss of this important neuromodulator under normoxic conditions. Interestingly, this apparently compensated network responded significantly different from wild-type (WT) mice, when exposed to anoxia.

METHODS

All electrophysiological experiments were performed on male and female mice (CD-1) of postnatal age 4–12 days. We used mice from two different breeding facilities: for the first set of experiments (dose-response curve and spantide experiments), we used a CD1 line that was bred in the animal facilities of the University of Chicago. For the experimental set including PPT-A mutant mice, mice were shipped from the laboratory of A. Basbaum at the University of California, San Francisco, CA. These PPT-A mutant mice have a disrupted gene for the SP and the NKA precursor: PPT-A (Cao et al. 1998). To control for possible differences in strains (see Tankersley et al. 1994), we used the WT of this line of CD-1 mice as the control.

Preparation

The mice were deeply anesthetized with ether, then decapitated at the spinal level of C8/C9. The preparation procedure has previously been described in detail (Ramirez et al. 1996), thus we will summarize only the most important steps. The brain was removed from the skull and immediately transferred into ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 118 NaCl, 3 KCl, 1.5 CaCl2, 1 MgCl2, 6 H2O, 25 NaHCO3, 1 NaH2PO4, and 30 n-glucose and equilibrated with carbogen (95% O2-5% CO2, pH 7.4). The brain stem was fixed on an agar block and secured in a vibratome with the rostral end up. Thin sections were sectioned serially from rostral to caudal until reaching the rostral boundary of the PBC. The level of the PBC was recognized by cytoarchitectonic landmarks, such as the absence of the facial nucleus and the presence of inferior olive (IO), nucleus of the solitary tract (NTS), hypoglossal nucleus (XII), and nucleus ambiguous (NA). The distance between the caudal end of the facial nucleus and the obex was ~700 µm in newborn mice. Portions of the VRG and XII were isolated in a 500- to 600-µm slice that was obtained ~200 µm caudal to the caudal end of the facial nucleus. The slice was immediately transferred into a recording chamber.

Submerged under a stream of ACSF (temperature, 29°C; flow rate, 11 ml/min), the preparation was stabilized for 30 min in ACSF. The potassium concentration in the ACSF was raised to 8 mM over a period of 30 min and maintained at this concentration to keep rhythmic activity patterns: a fast rhythmic activity (0.2 Hz), slower sigh activity, which consists of bursts with about two-three breaths per burst, and spantide activity, which we refer to as eupneic activity, and a superimposed periodic activity regular for the period of 30 min and maintained at this concentration to keep rhythmic activity regular for ≤13 h. The ACSF solution was recycled unless drugs were applied or washed out. Anoxia was induced by bubbling the ACSF with 95% N2-5% CO2 (pH 7.4). Exposure to anoxia was restricted to a period of 5 min.

Recording and data evaluation

Extracellular population activities of neurons in the PBC and surrounding VRG were recorded with electrodes that had an impedance of 120–150 kΩ when filled with ACSF. The electrodes were positioned with the visual aid of a binocular microscope (Zeiss, Axioskop) and the acoustic aid of a loudspeaker monitoring neuronal activity, which was evoked when touching the slice surface with the electrode. Signals were amplified (1,500 times), band-pass filtered (low-pass, 1.5 kHz, high-pass, 250 Hz), and electronically integrated (Paynter filter, set at a time constant of 40–50 ms; Fig. 1B, integrated traces). The data were digitized with a Digidata board (Axon Instruments), stored on a PC (Dell Pentium computer), and analyzed off-line with the software programs Axotape (Axon Instruments) and IGOR (WaveMetrics). Only recordings with good signal-to-noise ratios were analyzed (such recordings showed significantly larger amplitudes of integrated inspiratory activity than the variances of the noise and expiratory activity). Inspiratory bursts were detected by software programs using IGOR (WaveMetrics): after manual selection of a threshold, respiratory frequencies, burst duration, and rise time were calculated. Irregularity scores were determined for each cycle by applying the following formula for consecutive cycle length values: S = 100*ABS(Pr–Pn−1)/Pn with S the score of the nth cycle, Pn−1 being its period, Pn−1 the period of the preceding burst, ABS the absolute value (see Barthe and Clarac 1997). A low irregularity score therefore represents a regular rhythm. The higher the irregularity score the less regular the rhythm.

Drugs were bath applied at a final concentration of 10−7 to 10−5 M SP (Sigma, St. Louis, MO) and 5*10−6 M spantide (Peptides International, Louisville, KY). Slices were incubated in spantide for 2 h. Graphs were created in Prism (GraphPad Software). Data are presented as means ± SE. Significance was determined using Student’s t-test. Significance was assumed when P < 0.05.

RESULTS

Effect of SP on respiratory frequencies in control mice

Integrated extracellular recordings from cell populations (Fig. 1A, bottom) of the PBC and surrounding rostral portions of the VRG (Fig. 1A, top) reveal two types of fictive respiratory rhythmic activity patterns: a fast rhythmic activity (0.2 Hz), which we refer to as eupneic activity, and a superimposed slower sigh activity, which consists of bursts with about two-
fold larger amplitudes (marked as * in Fig. 1A) (for further characterization, see Lieske et al. 2000). Both eupneic and sigh frequencies were increased when the neuromodulator SP was bath applied (Fig. 1B). This is illustrated in dose-response curves that were obtained for both activities in control mice (Fig. 2). Concentrations as low as $10^{-9}$ M evoked a statistically significant effect on eupneic ($P = 0.08$) and sigh activity ($P = 0.01, n = 8$, Fig. 2). Responses to SP typically decreased after the initial maximal response, an effect that occurred faster at lower concentrations and that might be due to receptor desensitization or to endogenous peptidases. Averages for individual concentrations were therefore taken during the initial application of SP when the effect on frequencies was maximal (between 8 and 30 for eupnea and 3 and 10 for sighs). Beginning with the lowest SP concentration, we calculated the averages for three to four different concentrations per preparation. Following each application, SP was washed out for $10 \text{ min}$ until the frequencies returned to baseline values. The individual means were then averaged to receive the dose-response curves shown in Fig. 2 ($n = 8$). Note, that especially for higher concentrations of SP ($>10^{-8}$), the effect of SP was more pronounced on sigh frequencies than on eupneic activities. SP concentrations $>10^{-6}$ M evoked an additional and pronounced tonic activation, which often masked eupneic bursts completely. Therefore we did not determine the maximal saturating concentrations of SP.

**Effects of spantide on respiratory activity under normoxic conditions**

To investigate the role of endogenously released SP in the expression of these respiratory activities, we blocked SP receptors by bath application of 5 $\mu$M spantide (Fig. 3A). The mean eupneic frequency, evaluated by measuring consecutive cycle lengths during a 10-min episode, decreased significantly from $0.22 \pm 0.04$ Hz during control to $0.14 \pm 0.03$ Hz in

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**Fig. 1.** A: the acutely isolated slice preparation generates fictive respiratory activity that can be recorded extracellularly from cell populations in the pre-Bötzineger complex and ventral respiratory group (VRG, bottom). Top: the integrated activity of fictive respiration (Integr. VRG) with the expression of eupnea- and sigh-like (*) activities. All traces in the following figures are represented as integrated traces. Sp5, spinal trigeminal tract; XII, hypoglossal nucleus; NTS, nucleus tractus solitarius; NA, nucleus ambiguus; IO, inferior olive. B: substance P increases eupneic and sigh frequencies. At concentrations of $10^{-7}$ M application of substance P (bottom) induces in addition a tonic excitatory effect, reflected as a shift in the integrated baseline. At higher concentrations, this shift often masks the effect on eupneic activity.
Respiratory activity in PPT-A mutant in normoxia

PPT-A mutants were used as a model for a long-term deficiency of SP. To control for possible strain differences (see Tankersley et al. 1994), we used the WT of the same strain as control group. Respiratory activities in the WT were not significantly different from the mice raised in Chicago, with mean eupneic frequencies of $0.19 \pm 0.018$ Hz ($n = 20$) and $0.222 \pm 0.043$ Hz ($n = 9, P = 0.3$), and mean sigh frequencies of $3.7 \times 10^{-3}$ Hz $\pm 0.74 \times 10^{-3}$ ($n = 16$) and $3.63 \times 10^{-3} \pm 0.50 \times 10^{-3}$ ($n = 8, P = 0.75$), respectively.

Mean frequencies of both eupneic and sigh activities were not significantly different in the PPT-A mutant, although there was some variability between different slices (for extreme examples see Fig. 4A, middle and bottom). Mean values tended to be decreased in mutant mice [$0.16 \text{ Hz} \pm 0.02$ ($n = 29, P = 0.3$) for eupneic activities, Fig. 4B; and $3.1 \times 10^{-3} \text{ Hz} \pm 0.6$ ($n = 15, P = 0.56$) for sigh activities].

Despite individual variability in the regularity of respiratory activities (Fig. 4A), the analysis of mean regularity revealed that eupneic activity in the mutant mice was not significantly altered compared with WT mice ($t$-test: $P = 0.19, 31.38 \pm 4.7, n = 20$ WT; $47.2 \pm 5.8, n = 29$ PPT-A, Fig. 4C).

Effect of spantide on the anoxic augmentation

In naïve slices, anoxia leads to an increase in eupneic and sigh frequencies (anoxic augmentation), which usually peaks within 90 s and is followed by a depression of respiratory frequencies (see also Telgkamp and Ramirez 1999). To investigate a possible role of SP in modulating the anoxic augmentation, we induced anoxia in the presence and absence of the SP antagonist spantide (Fig. 5). To control for differences in respiratory baseline activities, we calculated the ratio between control frequency during normoxia and maximal frequency during anoxia (max frequency/control frequency). Control conditions were evaluated from a mean of $\sim 150$ eupneic cycles prior to the introduction of anoxic conditions. We induced two

![Graph showing dose-response curves of the effect of substance P on the frequency of eupneic and sigh activities.](http://jn.physiology.org/)
Anoxic augmentation in PPT-A mutant mice

The anoxic ratio was also evaluated in PPT-A mutant mice (Fig. 6). The WT mice from the same “breeder” were used as controls to ensure that possible differences were not due to differences in strain. Eupneic frequencies in the WT increased from 0.18 ± 0.02 Hz to values of 0.30 ± 0.02 Hz (n = 18). The anoxic ratio in the WT (1.9 ± 0.2; n = 18) was not significantly different from the values observed in the CD 1 mice bred in Chicago (see preceding text, 2.0 ± 0.3, n = 15, P = 0.76). The PPT-A mutant, however, showed a significantly higher anoxic ratio compared with the WT (4.1 ± 0.86, n = 15, P = 0.0116, Fig. 6B). Like for the experiments in spantide, we also determined the number of sighs during anoxic augmentation in PPT-A and WT mice. We observed a significantly lower number in the PPT-A mutant (1.04 ± 0.16, n = 28) compared with the WT (2.0 ± 0.33, n = 17, P = 0.005, Fig. 6C).

DISCUSSION

In the present study, we compared respiratory network activity after acute blockade of the SP receptor with the network activity in PPT-A mutant mice. Because PPT-A mutant mice lack the gene, which codes for the precursor of SP (Cao et al. 1998), this approach should reveal differences between short- and long-term effects of SP deprivation. We demonstrated that a blockade of endogenous SP receptors with spantide resulted in a significantly more irregular eupneic respiratory activity and a significantly decreased frequency of eupneic activity in the control mice. These findings suggest that endogenous concentrations of SP are involved in the control of the regularity and frequency of eupneic respiratory activity. In contrast, PPT-A mutant mice were on average unaffected, suggesting that these mice adapted to or compensated for the long-term deficiency in SP.

Compensation for the removal of neuromodulatory inputs has been demonstrated in studies of invertebrate rhythmic networks. Rhythm generation in these networks depends under control conditions on neuromodulatory inputs (Golowasch et al. 1999). After deprivation from modulatory inputs over several days, however, rhythmic activity reappeared and the network functioned in a “neuromodulator-independent state” (Golowasch et al. 1999; Thoby-Brisson and Simmers 1998). Indeed, regulatory mechanisms have been described that may be involved in readjusting membrane properties to the altered modulatory milieu (Gage et al. 1983; Terrigiano et al. 1994). Alternatively, the networks could adapt to missing neuromodulators by an alteration in the concentration of other endogenous modulators that are still present. Although not studied in a rhythm-generating network, there are several reports of compensatory mechanisms in genetically manipulated mice. For example a tenascin (TN)-mutant mouse shows significantly higher levels of PPT-A and cholecystokinin (CCK) mRNA in the terminal fields of dopaminergic neurons when compared with the WT (Fukamauchi and Kusakabe 1997). Here the alterations in other modulators could functionally compensate against the decreased level of the dopamine turnover rate.

One interesting aspect of the present study is that the compensatory mechanisms were obviously conditional. While respiratory activities were adjusted to the normoxic conditions, the response to anoxia was significantly different in the mutant mice. This suggests that adaptive properties of the respiratory system were altered after long-term deficiency in SP. This finding is reminiscent of the observation in children that suffer from Rett syndrome; these children exhibit significantly re-

![FIG. 4. A: integrated traces of respiratory activity in the WT (top) are comparable to respiratory activities in the control animals (see Fig. 3A). Respiratory activities in the preprotachykinin-A (PPT-A) mutant can vary considerably between different animals (extreme examples are shown in middle and bottom). B: mean eupneic frequencies, however, are not significantly altered in PPT-A mutants (WT: n = 20, PPT-A: n = 29, P = 0.3). C: irregularity scores in the PPT-A mutants are also not significantly increased (n = 20/29).](http://jn.physiology.org/)

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duced SP levels in the cerebrospinal fluid (Matsuishi et al. 1997) and a decreased SP immunoreactivity in the brain stem (Deguchi et al. 2000; Dunn and McLeod 2001). Under control conditions, the respiratory rate of these children is not different from healthy children (Kerr 1992; Kerr and Julu 1999). However, their breathing is maladaptive and these children exhibit frequently episodes of irregular respiratory frequencies and hyperventilation (Cirignotta et al. 1986; Lugaresi et al. 1985; Morton et al. 2000).

The role of SP in controlling the frequency and regularity of rhythmic activity resembles the situation previously described for the control of locomotor activity. Fictive locomotion can be induced with serotonin or NMDA in rat brain stem spinal cord preparations and in the lamprey swimming system. This rhythmic locomotor activity is often irregular and becomes regular only in the presence of SP (Barthe and Clarac 1997; Parker and Grillner 1998, 1999). Application of SP caused in these locomotor systems also an increase in the frequency of rhythmic

![Figure 5](image1.png)

**FIG. 5.** A: exposures to 5 min anoxia result in an anoxic augmentation in recordings of respiratory activities in the VRG both under control conditions and in the presence of 5 μM spantide. Both recordings shown here are from the same slice preparation. B: the anoxic ratio (max frequency in anoxia/control frequency) was determined for a 2nd exposure to anoxia in the control slices (control, n = 8) and for a 2nd exposure to anoxia in spantide (n = 7). C: the number of sighs during the anoxic augmentation were not significantly altered in the presence of spantide (P = 0.6, paired t-test).

![Figure 6](image2.png)

**FIG. 6.** A: integrated traces of respiratory activities during anoxic insults in wild-type (WT) and PPT-A mutant mice. The mutant exhibits a strong frequency augmentation on an anoxic insult. B: the ratio of the max frequency augmentation is significantly increased in the PPT-A mutant (n = 15/18, P = 0.012). C: the number of sighs is decreased in the PPT-A mutant (P = 0.005).
activity, which in case of the lamprey swimming system, even resulted in a nonreversible, long-lasting frequency increase after a 10-min exposure to SP (Parker and Grillner 1999; Parker et al. 1998).

SP is probably not the only neuromodulator that controls the frequency and regularity of rhythmic activity in the respiratory network. Other neuromodulators have been shown to be similar important. Serotonin modulates respiratory frequency via subtype-specific effects (Edwards et al. 1990; Lalley et al. 1994, 1995) and antagonists of serotonin receptors also lead to an irregular rhythmic activity under normoxic conditions (Morin et al. 1991). Thus the regularity and frequency of the respiratory activity seems to depend on a blend of neuromodulators, which include SP and serotonin. Minor differences in the relative endogenous concentrations of these neuromodulators will result in differences in the modulatory milieu. Such differences in the modulatory milieu could be responsible for differences in basal respiratory frequencies between different animal strains (Ptak and Hilaire 1999). They may similarly also explain strain differences in the response to hypoxic conditions (Tankersley et al. 1994), and it is interesting to note that there are also genetically manifested differences in how humans respond to hypoxia (Beall et al. 2000).

In conclusion, our study has demonstrated that in the transverse slice preparation endogenous SP has an excitatory effect on fictive eupneic activity under normoxic but not hypoxic conditions. Long-term deficiencies in SP, however, lead to compensatory mechanisms, which can adapt central respiratory activity to normoxic but not to hypoxic conditions. This finding is of great general interest as it indicates that long-term changes in the modulatory milieu may lead to compensatory mechanisms, which re-establish a baseline activity that is not significantly different from the normal network. At the same time, however, this compensated network may respond significantly different under stress conditions, such as hypoxia. This finding has also important clinical implications, as many disease states result in long-term changes in the modulatory milieu of neuronal networks. These networks may behave normally for most of the time, but abnormally when challenged by a stressful situation, such as the exposure to hypoxia.

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REFERENCES


