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# STIMULATION OF RAPHE (OBSCURUS) NUCLEUS CAUSES LONG-TERM POTENTIATION OF PHRENIC NERVE ACTIVITY IN CAT

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## SUMMARY

1. The respiratory response, measured as integrated phrenic nerve activity, during and for up to an hour following 10 min of continuous electrical stimulation of raphe obscurus was quantitated in anaesthetized, artificially ventilated cats whose carotid sinus nerves and vagus nerves had been cut. End-tidal $P_{CO_2}$ and body temperature were kept constant with servocontrollers.

2. Stimulation of raphe obscurus caused a significant increase in both phrenic tidal activity and respiratory frequency that persisted following cessation of the stimulus. This persistent facilitation is referred to as 'long-term potentiation' of respiration.

3. Control stimulations in the parenchyma of the medulla oblongata failed to stimulate respiration and cause the long-term potentiation.

4. Both the direct facilitatory effects of raphe obscurus stimulation on phrenic nerve activity and the long-term potentiation of respiration following the stimulus were prevented by pre-treating cats with methysergide, a serotonin receptor antagonist.

5. The results are discussed in terms of the raphe obscurus being the potential source of the long-term potentiation of respiration that occurs following stimulation of carotid body afferents (Millhorn, Eldridge & Waldrop, 1980*a*, *b*).

## INTRODUCTION

Brief stimulation (10 min) of carotid body chemoreceptor afferents, either physiologically or electrically, causes immediate facilitation of respiration and activates a central nervous system mechanism that continues to facilitate respiration for hours after cessation of the stimulus (Millhorn, Eldridge & Waldrop, 1980*a*). This prolonged stimulation of respiration is referred to as 'long-term potentiation.' Other inputs that facilitate respiration (central chemoreceptors and peripheral muscle) do not activate the mechanism responsible for the long-term potentiation. Thus the mechanism appears to be activated uniquely by afferent input from the carotid bodies. Since decerebration failed to prevent the long-term potentiation, the anatomical substrate for the mechanism must be located in the ponto-medullary region of the brain stem.

It was discovered subsequently (Millhorn, Eldridge & Waldrop, 1980*b*) that the

long-term potentiation of respiration was prevented by pre-treating animals with anti-serotonin agents (methysergide, parachlorophenylalanine and 5,7-dihydroxytryptamine), but not by pre-treating them with an inhibitor of catecholamine synthesis ($\alpha$-methyltyrosine). Since the major concentration of serotonin within the central nervous system is located in the brain-stem raphe nuclei (Dahlström & Fuxe, 1964; Poitras & Parent, 1978), it was hypothesized that the raphe nuclei are the anatomical substrate for the mechanism that causes the long-term potentiation.

The present study was undertaken to test this hypothesis. The effect on phrenic nerve activity during and after a relatively brief (10 min) episode of electrical stimulation of the obscurus region of the raphe nuclei was measured. To avoid interpretative problems associated with negative respiratory feed-back mechanisms, phrenic nerve activity was measured in anaesthetized, paralysed cats whose carotid sinus and vagus nerves had been cut and whose end-tidal $P_{CO_2}$ was servocontrolled. Stimulation of raphe obscurus always caused facilitation of phrenic nerve activity. The most important finding, however, was that phrenic activity remained elevated above the original control level for more than an hour after termination of the stimulus. Both the direct facilitatory effect of the stimulus and the long-term potentiation were prevented by pre-treating animals with methysergide.

## METHODS

Adult cats ($n = 13$) weighing between 1·5 and 3·7 kg were studied. They were anaesthetized initially with ether and then given chloralose (40 mg/kg) and urethane (250 mg/kg) via a femoral vein. Femoral arterial pressure was measured by means of a strain gauge. Temperature was monitored by a rectal thermistor and servocontrolled at 37·5 °C by an electronic circuit and d.c. heating pad. The trachea was cannulated. Airway gas was sampled through the catheter. Analysis of airway $P_{CO_2}$ was performed by an infra-red analyser.

Both vago-sympathetic trunks were isolated and cut in the cervical region of the neck. The carotid sinus nerves were identified and cut. The animal's head was then placed in a stereotaxic apparatus (Kopf) and a posterior fossa craniotomy performed. One phrenic nerve root (C5) was isolated from the lateral approach, cut distally, desheathed and placed on a bipolar platinum recording electrode. The electrode was built into a small acrylic platform that was placed in a tissue well adjacent to the nerve. Because it has no firm external attachments (the electrical wires were flexible and moved freely), it was possible even during a long experiment to maintain a relatively constant coupling between nerve and electrode. Both nerve and electrode were immersed in mineral oil.

Following these preparations the animals were paralysed with gallamine triethiodide (3 mg/kg I.V. initially) followed by a continuous infusion at a rate of 3 mg/kg . h to maintain paralysis. They were ventilated with 100 % $O_2$ by means of a volume-cycled ventilator (Harvard). In order to prevent significant changes in end-tidal and arterial $P_{CO_2}$ secondary to changes in cardiac output and venous return to the lungs, the d.c. voltage on the ventilator's motor was controlled by the animal's end-tidal $P_{CO_2}$ level through an electronic circuit (Smith, Mercer & Eldridge, 1978). The ventilator's rate was thereby servocontrolled to maintain $P_{CO_2}$ constant. The combination of chloralose and urethane provides a very long-lasting anaesthesia. In a previous study (Eldridge, 1973) where muscle paralysis was not included, no awakening or evidence of pain sensitivity occurred during a 6 or 7 h experiment. Inasmuch as the time course of the present experiments was similar, no additional anaesthesia was given.

At this point, the dura overlying the medulla and caudal cerebellum was cut and retracted laterally. In order to expose the dorsal surface of the medulla, the caudal part of the cerebellum was aspirated. A stainless-steel stimulating electrode (monopolar, 100 KΩ) was placed in a micromanipulator (Kopf) and positioned perpendicular to the surface of the medulla and aligned visually with the mid line at the level of the obex. Square-wave pulses of constant current were

delivered via a stimulator (Grass S88) connected in series with a stimulus isolation unit and fixed resistance. The parameters for electrical stimulation ranged from 15 to 100 $\mu$A, 25 to 50 Hz, with pulse durations of 50–100 $\mu$s. The amplitude of the pulses was adjusted so as to be sufficient to cause facilitation of phrenic nerve activity during the stimulation period.

*Experimental protocol*

The raphe is a narrow structure (0·7–1·2 mm) located at the mid line of the brain stem. The topological map of the raphe nuclei compiled by Taber, Brodal & Walberg (1960) was used to locate the obscurus region. The stimulating electrode was positioned on the mid line and 0–1 mm rostral to the obex. The tip was positioned at a depth of 2–3 mm beneath the dorsal surface. Prior to stimulation of raphe obscurus ($n = 7$), a control stimulation was performed with the same stimulation parameters and at the same coordinates but 2–3 mm lateral to the mid line. This was done to rule out current spread to nuclei within the medulla that are traditionally associated with respiration. In order to verify the raphe stimulation site, a d.c. lesioning current was applied at the end of the experiment. The animal was perfused transcardially with 10% formalin solution, the brain removed and processed for histology.

The regime was the same for both the 'off mid line' and raphe stimulations. When all variables had become stable, control values were recorded for a period of 3 min. The stimulation was then given continuously for 10 min, with the last 3 min recorded. The responses during the initial 7 min were essentially the same as those recorded. At the end of this period the stimulus was stopped and recordings of all variables made at 5 min intervals for up to 30 min. In several cases recovery was followed for 1 h.

Another group of animals ($n = 6$) was given methysergide (1 mg/kg), a specific serotonin receptor antagonist (Gorlitz & Frey, 1973; McCall & Aghajanian, 1980) at least 30 min before raphe stimulation. The agent was administered intravenously in 3 ml Ringer solution. This always led to a decrease in phrenic activity and arterial pressure (Millhorn *et al.* 1980*b*). End-tidal $P_{CO_2}$ was increased in order to increase phrenic activity to the level measured before administration of the drug. When the variables had once again become stable, the stimulation protocol outlined above was performed.

*Data analysis*

Data obtained included arterial pressure, phrenic impulses, airway $CO_2$ and a stimulus marker; all were recorded on magnetic tape and on a direct writing recorder (Gould). On-line analysis was performed with a computer (DEC 11/23). Integrated phrenic nerve activity was determined for each 100 ms period by means of a step-hold integrator (Gould). Since the peak integrated value of phrenic activity is a satisfactory index of inspiratory (tidal) activity (Eldridge, 1975), neural (phrenic) minute activity was computed as the product of phrenic tidal activity and respiratory frequency.

In order to compare findings among different animals, the data were normalized by assigning a value of 85 arbitrary units to the highest level of phrenic activity measured in an individual cat when end-tidal $P_{CO_2}$ had been increased 40 torr above threshold. The basis of this technique of normalization was reported recently by Eldridge, Gill-Kumar & Millhorn (1981). Analysis of variance was used to determine if values during stimulation and 30 min after stimulation were different from control values. Differences were considered significant at a $P < 0·05$ level.

## RESULTS

Fig. 1 shows the effect on integrated phrenic nerve activity of electrical stimulation of raphe (obscurus) nuclei in one cat. End-tidal $P_{CO_2}$ was kept constant (32 torr) during the entire experiment. Following the control period, the stimulus was applied continuously for 10 min (20 $\mu$A, 50 Hz, 50 $\mu$s). This led to an increase in phrenic activity. Following cessation of the stimulus, there was an initial augmentation of phrenic activity. The activity then remained well above the original control level for the remainder of the recovery period (1 h). Arterial pressure was not affected by the stimulus.

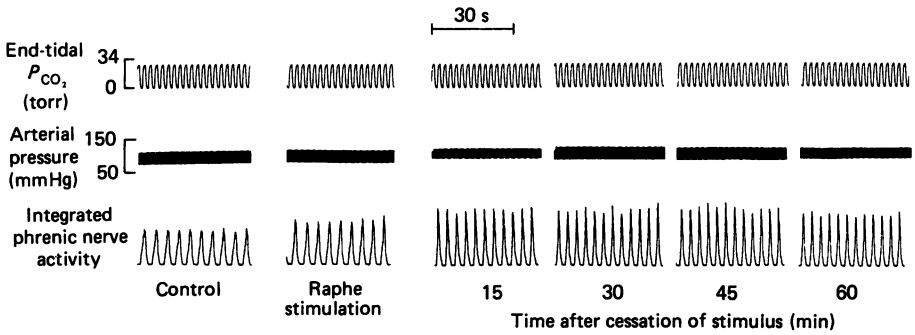


Fig. 1. Effect on phrenic nerve activity, arterial pressure and end-tidal $P_{CO_2}$ during and following electrical stimulation of raphe obscurus in one cat. The stimulation parameters are given in the text. Control levels for all variables are shown at the left.

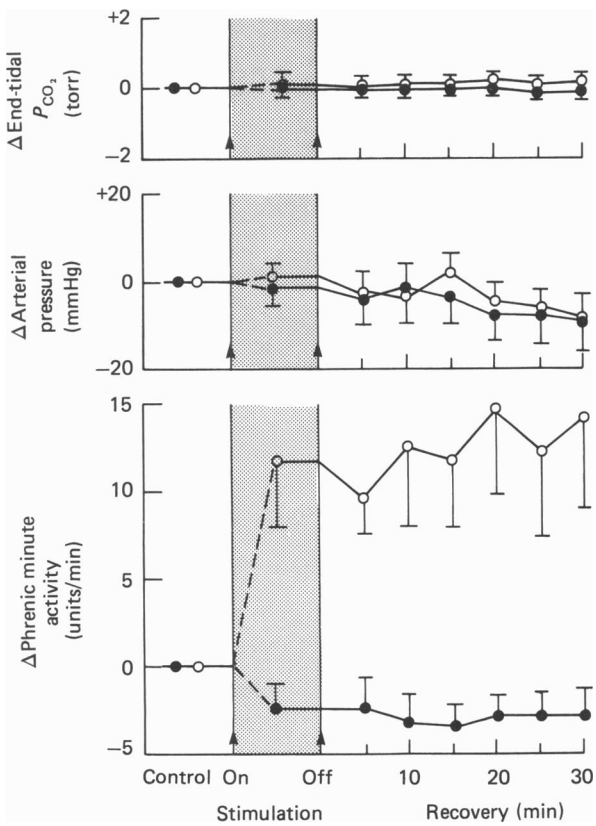


Fig. 2. Averaged effects on phrenic minute activity, arterial pressure and end-tidal $P_{CO_2}$ of raphe obscurus stimulation (open circles) in seven cats and 'off mid line' stimulation (filled circles) in seven cats. The data are presented as change from control which is shown at the left. The stippled area represents the last 3 min of the 10 min stimulus period. The recovery values were measured for 1 min at 5 min intervals. Values are means ± S.E. of mean.

The averaged findings for seven cats studied this way (open circles) are given in Fig. 2. The stimulation always caused an increase in phrenic minute activity. In order to decrease variability of the nerve recording that might result from a changing relationship between the phrenic nerve and electrode, the recovery period was limited to 30 min. Following offset of the stimulation, phrenic minute activity remained

TABLE 1. Phrenic activity, arterial pressure and end-tidal $P_{CO_2}$ before, during and after raphe stimulation ($n = 7$)

| | Control | Raphe stimulation | 30 min after raphe stimulation |
|---|---|---|---|
| Phrenic tidal activity (units) | 29·0 ± 0·80 | 39·0* ± 3·5 | 39·9* ± 3·5 |
| Respiratory frequency (breaths/min) | 10·6 ± 1·0 | 13·9* ± 2·1 | 13·6* ± 1·9 |
| Phrenic minute activity (units/min) | 25·4 ± 2·5 | 39·0* ± 3·9 | 43·0* ± 2·5 |
| Arterial pressure (mmHg) | 100·0 ± 7·9 | 102·0 ± 9·4 | 95·0 ± 5·1 |
| End-tidal $P_{CO_2}$ (torr) | 34·4 ± 1·9 | 34·6 ± 1·9 | 34·6 ± 1·9 |

Values are means ± S.E. of mean.
* Denotes significant difference ($P < 0·05$) from control.

significantly elevated above the original control level for the entire recovery period. This long-term potentiation of phrenic activity cannot be explained by an increase in end-tidal $P_{CO_2}$, which remained constant. Arterial pressure did not change during the stimulation period but did show a slight hypotension during the recovery period. The same intensity stimulation was applied before raphe stimulation at the same depth but 2–3 mm lateral to the mid line. Averaged findings ($n = 7$) from these experiments (filled circles) are also given in Fig. 2. In some animals the stimulation had an immediate facilitatory effect on phrenic activity, whereas in others it caused inhibition; the averaged result was a slight inhibition. Following termination of the stimulus, phrenic activity remained below the original control level during the entire recovery period. In other words, stimulation at sites off the mid line did not cause long-term potentiation of respiration. Arterial pressure showed a decrease that was identical to that measured after raphe stimulation. The actual values for all variables measured during control, raphe stimulation and 30 min after raphe stimulation are given in Table 1.

In order to determine if the long-term potentiation measured following stimulation of raphe obscurus were due to release of serotonin, a separate group of cats was studied after pre-treatment with methysergide, a serotonin receptor antagonist (Gorlitz & Frey, 1973; McCall & Aghajanian, 1980). The findings from one such experiment are shown in Fig. 3. The stimulation (50 $\mu$A, 50 Hz, 50 $\mu$s) had no direct facilitatory effect on phrenic nerve activity or, more important, the level of activity following cessation of the stimulus. Arterial pressure during the stimulation and recovery periods was essentially unchanged from the control level. End-tidal $P_{CO_2}$ was kept constant during the entire experiment.

The averaged findings from six cats pre-treated with methysergide (filled squares) are given in Fig. 4 and are compared with those from the animals that did not receive methysergide (open circles). The data from the non-treated animals ($n = 7$) are the same as those shown for raphe stimulation in Fig. 2. It is clear that methysergide

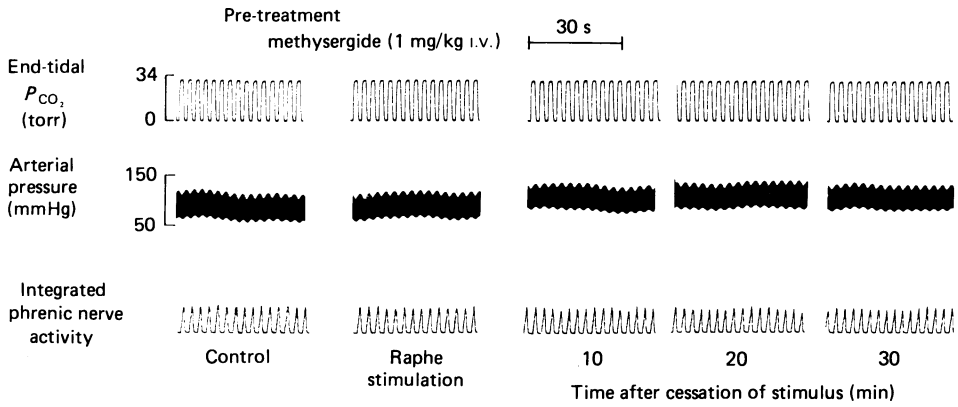


Fig. 3. Effect on phrenic nerve activity, arterial pressure and end-tidal $P_{CO_2}$ during and following electrical stimulation of raphe obscurus in a cat that had been pre-treated with methysergide. The stimulation parameters are given in the text. Control levels for all variables are shown at the left.

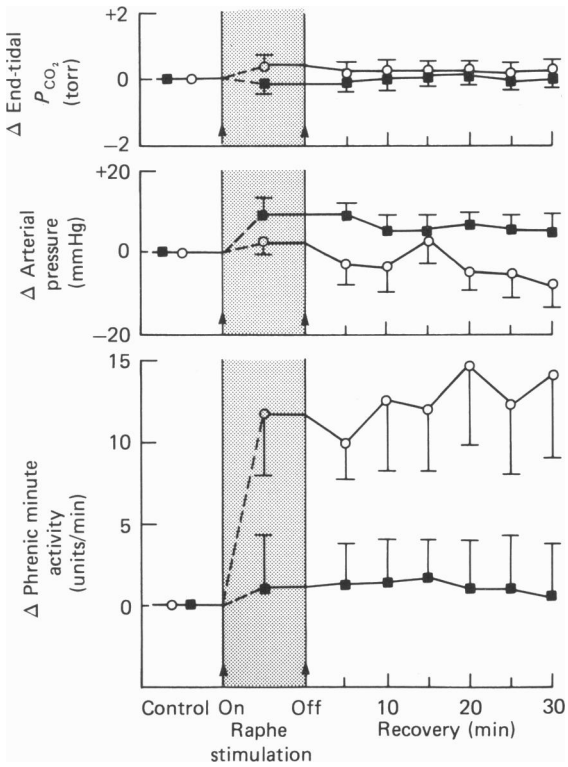


Fig. 4. Averaged effects on phrenic minute activity, arterial pressure and end-tidal $P_{CO_2}$ of raphe obscurus stimulation in seven non-treated (open circles) and six methysergide pre-treated (filled squares) cats. The data are presented as change from control which is shown at the left. The stippled area represents the last 3 min of the 10 min stimulus period. The recovery values are measured for 1 min at 5 min intervals. Values are means $\pm$ S.E. of mean.

prevented both the direct facilitatory effect on phrenic activity and the long-term potentiation of phrenic nerve activity following raphe stimulation. This finding supports serotonin being responsible for mediating the long-term potentiation of respiration following raphe stimulation.

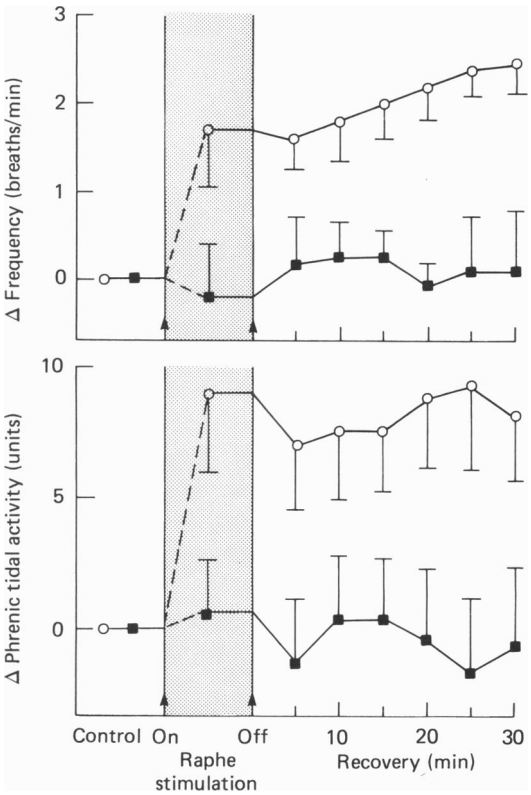


Fig. 5. Averaged effects on phrenic tidal activity and respiratory frequency of raphe obscurus stimulation in the seven non-treated (open circles) and six methysergide pre-treated (filled squares) cats. The data are presented as change from control which is shown at the left. Values are means ± S.E. of means.

Fig. 5 shows the effect on phrenic tidal activity and respiratory frequency for both the methysergide pre-treated group (filled squares) and the non-treated group (open circles). It is important to note that the long-term potentiation of phrenic minute activity in the non-treated animals was due to significant increases in both phrenic tidal activity and respiratory frequency. Neither variable showed a significant increase in the group pre-treated with methysergide. The actual values for all variables measured during control, raphe stimulation and 30 min after raphe stimulation in the methysergide pre-treated cats are given in Table 2.

TABLE 2. Phrenic activity, arterial pressure and end-tidal $P_{CO_2}$ before, during and after raphe stimulation in methysergide pre-treated cats ($n = 6$)

| | Control | Raphe stimulation | 30 min after raphe stimulation |
|---|---|---|---|
| Phrenic tidal activity (units) | 32·3 ± 2·8 | 32·7 ± 3·0 | 31·5 ± 2·9 |
| Respiratory frequency (breaths/min) | 20·7 ± 1·1 | 20·3 ± 1·2 | 20·8 ± 1·1 |
| Phrenic minute activity (units/min) | 30·2 ± 2·2 | 30·7 ± 3·8 | 30·8 ± 4·3 |
| Arterial pressure (mmHg) | 67·0 ± 6·2 | 76·0 ± 9·3 | 72·0 ± 6·1 |
| End-tidal $P_{CO_2}$ (torr) | 33·3 ± 0·8 | 33·1 ± 0·7 | 33·0 ± 0·8 |

Values are means ± S.E. of mean.
The values during stimulation and 30 min after stimulation were not different from control values.

## DISCUSSION

In earlier studies, Millhorn *et al.* (1980*a*, *b*) reported that in addition to the immediate facilitatory effects on respiration that result from stimulation of carotid body afferents, a central serotoninergic mechanism is activated that continues to facilitate respiration for hours after cessation of the original stimulus. This persistent facilitation is referred to as 'long-term potentiation' of respiration. The present study was undertaken to determine if stimulation of the raphe nuclei, the primary site of serotonin within the central nervous system (Dahlström & Fuxe, 1964; Poitras & Parent, 1978), would cause long-term potentiation of respiration similar to that measured following carotid body stimulation. I found that a relatively brief episode (10 min) of electrical stimulation of the caudal portion of raphe obscurus caused facilitation of respiration that lasted for more than an hour after the stimulus had been terminated. The magnitude of the long-lasting facilitation was almost identical to that measured following carotid body afferent stimulation in the aforementioned studies (Millhorn *et al.* 1980*a*, *b*). Because the response was prevented by methysergide, a specific serotonin receptor antagonist (Gorlitz & Frey, 1973), I conclude that both the immediate effect and long-term potentiation of phrenic nerve activity in response to stimulation of raphe obscurus are mediated by serotonin.

The present study was not designed to study the effect on phrenic nerve activity of stimulation of sites throughout the entire raphe complex. Instead, I concentrated on the obscurus subnucleus because electrical stimulation of this site appears to be more consistent in eliciting an excitatory effect on arterial pressure (Adair, Hamilton, Scappaticci, Helke & Gillis, 1977) and phrenic nerve activity (Holtman, Anastasi, Norman and Dretchen, 1986*a*; Holtman, Dick & Berger, 1986*b*) than any other subnuclei of the raphe complex. It is entirely possible though that stimulation of the other caudal subnuclei (magnus and pallidus) would also stimulate respiration (Dretchen, Holtman, Hamilton, Norman & Gillis, 1983; Lalley, 1985) and cause long-term potentiation of phrenic activity.

Because the raphe complex is a narrow structure (0·7–1·2 mm), current spread into neighbouring medullary parenchyma is entirely possible. In order to rule out current spread as being responsible for the results, I stimulated sites (2–3 mm lateral to the mid line) near areas traditionally associated with respiration. In no case did this cause either direct stimulation of phrenic nerve activity or the long-term potentiation.

I was also concerned that the measured responses during and after raphe stimulation might be due to activation of descending respiratory efferents that are known to decussate close to the stimulation site (Lipski, Kubin & Jodkowski, 1983). Evidence that this was not the case comes from my finding that respiratory frequency is increased during stimulation and remains elevated throughout the recovery period. In order for respiratory frequency to increase, the stimulus must have affected input to the respiratory rhythm generator. Additional evidence that phrenic nerve responses to stimulation of raphe obscurus are not due to activation of axons of passage comes from the work of Holtman *et al.* (1986*a*) which showed that excitation of cell bodies within raphe obscurus by microinjections of L-glutamate, an excitatory amino acid, caused facilitation of phrenic nerve activity.

Holtman *et al.* (1986*b*) proposed that serotonin-containing axons from raphe obscurus travel to respiratory related areas within the brain stem and then to the phrenic motonucleus by the same pathway(s) that carries signals from the respiratory rhythm generator. They found that microstimulation of raphe obscurus led to an early (2·5 ms) as well as a delayed (7 ms) excitation of phrenic motoneurones. The delayed but not the early excitation was prevented by pre-treatment with serotonin receptor antagonists. The early excitation is thought to be due to stimulation of descending axons from bulbo-spinal inspiratory neurones that cross the mid line near the site of stimulation (Lipski *et al.* 1983). The delayed excitation, on the other hand, is believed to be due to activation of serotonin-containing neurones in raphe obscurus that project to respiratory related areas. The present finding that the excitatory effect on phrenic nerve activity during and after stimulation of raphe obscurus was in part due to an increased respiratory frequency suggests that input from raphe obscurus affects the respiratory rhythm generator.

Additional evidence that the raphe nuclei send projections to areas within the medulla associated with respiration comes from anatomical and histochemical studies of brain-stem nuclei. Palkovits, Brownstern & Saavedra (1974) reported the existence of serotonin-containing pathways between the raphe complex and the nucleus of the tractus solitarius (i.e. the dorsal respiratory group). Steinbusch (1981) found serotonin nerve terminals in the vicinity of the nucleus ambiguus (i.e. the ventral respiratory group).

If the raphe nuclei are involved in mediating the long-term potentiation of respiration that occurs following stimulation of carotid body afferents (Millhorn *et al.* 1980*a*, *b*), there must be a pathway connecting the carotid bodies with medullary raphe nuclei. Evidence for such a pathway is based on the electrophysiological studies of Humphrey (1967) and Miura & Reis (1969). These workers showed that evoked potentials could be recorded within the medullary raphe nuclei in response to electrical stimulation of the carotid sinus nerve. Since the whole carotid sinus nerve was stimulated, it is impossible to determine if the evoked potentials were due to activation of baroreceptor or chemoreceptor afferents. Thus, the evidence for such a pathway is relatively weak.

The concept of long-term potentiation of neural activity is not new. Bliss & Lomo (1973) reported that repetitive volleys of electrical stimulation of fibres within the perforant pathway caused excitation of hippocampal cells that lasted from 30 min to 10 h following cessation of the stimulus. They believed that the long-term

potentiation of neuronal activity could best be explained by (1) an increase in the efficiency of synaptic transmission, or (2) an increase in the excitability (i.e. sensitization) of the post-synaptic cell. Hökfelt, Johansson & Goldstein (1984) recently proposed a model of synaptic transmission that offers an explanation for long-term potentiation of neural activity within the central nervous system. Their model is predicated on the coexistence of a classical neurotransmitter and one or more peptides within single nerve terminals. The classical transmitter, and perhaps a peptide, are concerned primarily with transmission of signals between the pre- and post-synaptic elements. One peptide is thought to be responsible for prolonging the release of the classical neurotransmitter (and therefore the post-synaptic response) by inhibiting 'autoregulators' located in the presynaptic membrane. The autoregulators are activated by the presence of the classical transmitter in the synaptic cleft and once activated cause a decrease or cessation of the release of the neurotransmitter. If the autoregulators are inhibited, synaptic transmission continues indefinitely and the post-synaptic element would show long-term potentiation.

There is evidence that serotonin coexists in some nerve terminals within the medulla oblongata with substance P and thyrotropin-releasing hormone (TRH) (Chan-Palay, Jonsson & Palay, 1978; Hökfelt, Ljungdahl, Steinbusch, Verhofstad, Nilsson, Brodin, Pernow & Goldstein, 1978). In this scheme, serotonin and TRH are responsible for transmission of impulses between the pre- and post-synaptic elements and substance P causes inhibition of the presynaptic autoregulator.

*Conclusion*

The present study confirms that stimulation of raphe obscurus causes immediate facilitation of phrenic nerve activity. A new finding is that brief (10 min) stimulation of raphe obscurus activates a serotoninergic mechanism that continues to stimulate phrenic activity for more than an hour after cessation of the stimulus. The functional significance of this mechanism was not elucidated by the present study. In an earlier study, Millhorn *et al.* (1980*b*) reported the existence of a similar long-lasting serotoninergic mechanism that was activated by brief stimulation of carotid body afferents. I believe that the raphe nuclei are a component of that mechanism. The long-term potentiation found following raphe obscurus stimulation was essentially identical in both time course and magnitude as that found following carotid body afferent stimulation (Millhorn *et al.* 1980*a*).

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