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NORADRENERGIC MODULATION OF THE MEDULLARY RESPIRATORY RHYTHM GENERATOR IN THE NEWBORN RAT: AN *IN VITRO* STUDY

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SUMMARY

1. Superfused brain stem–spinal cord preparations of newborn rats, which continue to show a rhythmic respiratory activity *in vitro*, were used to analyse the mechanisms whereby the A5 noradrenergic area modulates the activity of the medullary respiratory rhythm generator in the newborn.

2. In preparations including the pons (ponto-medullary preparations), noradrenaline (NA, 25–100 μM) added to the bathing medium either increased ($n = 29/50$) or decreased ($n = 21/50$) the respiratory frequency and elicited a tonic discharge in the cervical ventral roots in 50% of the experiments. Double-bath experiments showed that the increases in respiratory frequency were due to NA acting on the pons, whereas the decreases in respiratory frequency were due to NA acting on the medulla. The NA-induced increases in respiratory frequency were attributed to inhibition of A5 neurons by NA and therefore to withdrawal of A5 inhibition on the medullary rhythm respiratory generator. The NA-induced decreases in respiratory frequency seemed to mimic the effects of endogenous NA on the A5 medullary targets.

3. Noradrenaline-induced tonic activity (i) could be induced after elimination of the pons but not on isolated spinal cord, (ii) could be elicited by α_1- but not α_2-agonists, (iii) could be blocked by α_1- but not α_2-antagonists. The tonic activity therefore originated from activation of α_1 receptors located in the medulla but its importance in respiratory function is doubtful.

4. In medullary preparations (elimination of the pons by transection), the effects of NA agonists and antagonists on respiratory frequency were analysed. Significant decreases in respiratory frequency were induced by NA, adrenaline, phenylephrine and α-methyl-NA, but not by the agonists classified as α_2 (clonidine and guanfacine), α_1 (6-fluoro-NA) and β (isoprenaline). Since yohimbine, idazoxan and piperoxane (α_2 antagonists) blocked the NA-induced decreases in respiratory frequency whereas prazosin (α_1 -antagonist) did not, it is postulated that α_2-receptors may be involved in modulating respiratory frequency.

5. Stimulation, lesion and NA microejection experiments showed the complexity of the mechanisms mediating NA-induced changes in respiratory activity but

suggested that the main site of NA action is located in the rostral ventrolateral medulla, where electrical stimulations triggered inspiration prematurely, lesions suppressed the NA-induced decrease in respiratory frequency, and localized application of NA led to an immediate decrease in the respiratory frequency.

6. To conclude, the A5 noradrenergic modulation of the medullary respiratory rhythm generator may be relayed by α_2-adrenoceptors located within the rostral ventrolateral medulla. Discrepancies between the respiratory effects elicited by NA and α_2-agonists raise the question as to whether these drugs are actually potent agonists, or whether the adrenoceptors are functionally mature at birth.

INTRODUCTION

The regulation of the cardiovascular and respiratory systems is based on mechanisms which interact peripherally and/or centrally: any changes in cardiovascular function may induce changes in respiratory-related variables (pulmonary gas exchanges, baroreceptor and chemoreceptor afferents, activities of the cardiovascular 'centres', etc.), which in turn may give rise to central respiratory changes. These interactions have made it difficult to determine whether the central structures involved in the regulation of these two systems constitute a functional entity. Some neurons are affected not only by cardiovascular changes but also by ventilatory changes, and it is often difficult to define whether these influences arise from the periphery or not, and whether these neurons are primarily cardiovascular or respiratory neurons (Haselton & Guyenet, 1989).

Suzue (1984) demonstrated that the isolated brain stem preparation of newborn rats retains the ability to produce spontaneous central respiratory activity *in vitro*. This reduced preparation constitutes a useful tool for analysing the pharmacology of the respiratory centres at birth, since the possibility of peripheral effects can be eliminated. It was demonstrated previously that in this preparation the pontine A5 area modulates the activity of the medullary respiratory rhythm generator at birth (Hilaire, Monteau & Errchidi, 1989). Using stimulation, lesion and pharmacological experiments, A5 has been shown to tonically depress the medullary respiratory rhythm generator (Hilaire *et al.* 1989) via a permanent release of noradrenaline (NA) originating from A5 which slows down the respiratory frequency (RF) (Errchidi, Hilaire & Monteau, 1990). The A5 area, which is known to be involved in cardiovascular regulation (Andrade & Aghajanian, 1982; Guyenet, 1984; Byrum & Guyenet, 1987), may also be involved in respiratory regulation, at least at birth.

The aim of the present study was to analyse more thoroughly the A5 modulation of the medullary respiratory rhythm generator, and in particular to define the type and location of the adrenoceptors involved. Results were obtained suggesting that α_2-receptors located in the rostral ventrolateral medulla may be involved. The question arises, however, as to how mature the α_2-receptors may be at birth and/or whether some of the compounds classified as α_2-agonists are actually potent agonists.

METHODS

Newborn rats (0–4 days old) were anaesthetized with ether and decerebrated. A section was performed at the level of the last cervical vertebra and the skin and muscles were rapidly removed. The dissection was continued in a 2 ml chamber filled with artificial cerebrospinal fluid identical to

that previously used (Hilaire *et al.* 1989), equilibrated with 95% O_2 and 5% CO_2, warmed to 27 ± 0.5 °C, and permanently drained by suction with a perfusion rate such that the total chamber volume was replaced within 30 s. After removal of the cranial and vertebral bones under binocular control, the brain stem was fixed with the ventral surface upwards.

The inspiratory electrical activity of either the cervical ventral roots or the hypoglossal roots was recorded using suction electrodes. The signals were filtered (5–3000 Hz), amplified and fed to a leaky integrator (time constant, 100 ms), oscilloscope and paper recorder. The intervals between the bursts of potentials on the records were used to define the RF (Fig. 1).

Electrical stimulations (single shock, 0·3 ms in duration, 25–100 μA) were performed with a digital stimulator via an isolation unit (WPI 830) and either thin tungsten microelectrodes (Frederick Haert, impedance 5–10 MΩ) or the central barrel of multibarrelled micropipettes filled with the bathing medium (impedance 2–5 MΩ). Electrocoagulations were performed with long duration DC current (100–200 μA, 2–5 s). Microelectrodes and micropipettes were moved by a motor drive micromanipulator capable of 1 μm steps. After fixation of the brain stem in 2–4% glutaraldehyde, frozen sections (thickness 100 μm) were cut and examined histologically to plot the sites marked by electrocoagulation.

The following drugs (RBI and Sigma) were used as pharmacological tools: clonidine, α-methyl-NA and guanfacine as α_2-agonists (Cedarbaum & Aghajanian, 1977; Jarrot, Louis & Summers, 1982), 6-fluoro-NA as α_1-agonist (Chiueh, Zukowska-Gorjec, Kirk & Kopin, 1983), phenylephrine (as mainly α_1- but also α_2-agonist; Cedarbaum & Aghajanian, 1977), isoprenaline as β agonist, piperoxane, yohimbine, idazoxan as α_2-antagonists (Cedarbaum & Aghajanian, 1977; Freedman & Aghajanian, 1984), and prazosin as α_1-antagonist (U'Prichard, 1981). Drugs were dissolved in the bathing medium and applied by superfusion for 6–9 min. Occasionally, the *in vitro* bath was partitioned with a barrier (improved with Vaseline) at the ponto-medullary junction (double-headed arrow in Fig. 1) so that the pons and the medulla could each be superfused with a different medium. In each experiment, a control period of at least 4 min was used to define the mean respiratory frequency per minute under normal medium. The preparation was then superfused with a medium containing drug and the resulting changes were expressed each minute as a percentage of the mean control value. In some experiments, multibarrelled micropipettes were used to apply drugs locally to the brain stem (pressure pulses). Prior to the experiments, control ejections were performed under the microscope: the ejected volumes never exceeded 5 nl. Experiments were repeated on several brain stem preparations with a standardized procedure to evaluate the mean effects. Results were expressed as the mean ± S.E.M. and any differences were tested using Student's t test, and were taken to be significant at P values lower than 0·05.

RESULTS

Ponto-medullary preparations

In ponto-medullary preparations ($n = 109$), the resting respiratory frequency (RF) was around 4–5 cycle min^{-1}, and showed only restricted variations (less than 10% of the control values) during the experiment. A significant difference was observed, however, between the mean RF of 0 to 1-day-old rats ($3{\cdot}8 \pm 0{\cdot}6$ cycle min^{-1}, $n = 50$) and that of 2 to 4-day-old rats ($5{\cdot}5 \pm 0{\cdot}6$ cycle min^{-1}, $n = 59$).

Replacing the normal medium by a medium containing NA (10–100 μM) resulted in either an increase (Fig. 1*B*; 29/50 experiments; mean increase 66% of control value) or a decrease (Fig. 1*C*; 21/50 experiments; mean decrease 32% of control value) in the RF. In a given preparation, NA always elicited the same change if tested repetitively. Respiratory modification occurred with a latency of 2–3 min and reached a maximum within 6–9 min. Whatever type of RF change occurred, a simultaneous tonic activation was recorded in half of the cases with a 3–5 min latency in all the cervical ventral roots but never in hypoglossal nerves (Fig. 1*B*). The respiratory activity returned to control values 6–10 min after wash-out of the NA medium.

A barrier was placed at the ponto-medullary junction (see the double-headed

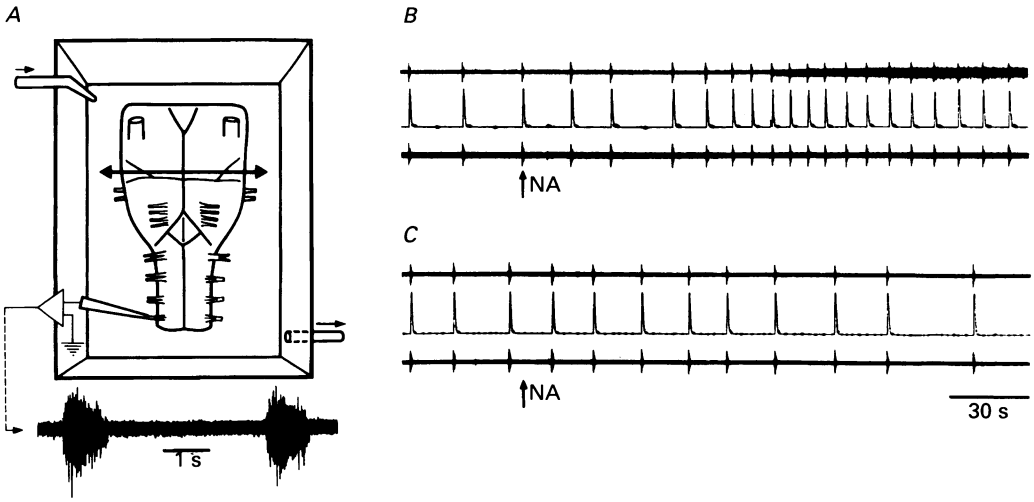

Fig. 1. Respiratory changes induced by NA bathing in ponto-medullary preparations of newborn rat. *A*, schematic drawing of the newborn brain stem–spinal cord preparation showing the pattern of discharge recorded in a cervical ventral root; the horizontal double-headed arrow indicates the level of the barrier (in double-bath experiment) and the level of transection (to eliminate the pons in medullary preparations). *B* and *C*, from top to bottom, activity of a cervical ventral root, integrated and raw activity of the hypoglossal nerve; the two records correspond to two different preparations. Replacing the normal bathing medium by a medium containing NA (at the arrow) increased the respiratory frequency (RF) and elicited a permanent discharge in the cervical ventral root but not in the hypoglossal nerve in one preparation (*B*) and decreased the RF in another (*C*). Time as indicated.

TABLE 1. Changes in respiratory frequency induced by media containing different concentrations of NA applied to either the pons or the medulla

[NA]	5 μM	10 μM	25 μM	100 μM
Applied to the pons	n.s.	+13±4	+36±13	+66±14
Applied to the medulla	n.s.	−21±4	−28±3	−36±5

All values are means±S.E.M. expressed as a percentage of controls ($n = 6$). Bathing with medium containing 5 μM-NA evoked non-significant changes: n.s.

arrow in Fig. 1*A*) in order to apply various media to either the pons or the medulla. When the pons was bathed with a medium containing NA and the medulla with a normal medium, the RF increased ($n = 6/6$). In contrast, when the medulla was bathed with medium containing NA and the pons with normal medium, the RF decreased ($n = 6/6$). A dose-dependent relationship was observed under both experimental conditions (see Table 1). The α_2-agonist clonidine applied to the pons had NA agonist effects since it significantly increased the RF at concentrations ranging from 25 to 100 μM (26±13%, $n = 10$) but had no effect at lower concentrations (10 μM, $n = 5$). Surprisingly, clonidine did not significantly change the RF when applied to the medulla (10–100 μM, $n = 15$) and never elicited any tonic discharge in cervical roots.

Medullary preparations

The effects of NA were then analysed in medullary preparations (elimination of the pons by transection at the level indicated by the double-headed arrow in Fig. 1*A*, $n = 95$). As already described (Hilaire *et al.* 1989; Errchidi *et al.* 1990), ponto-medullary transection did not suppress the respiratory activity but immediately induced a sustained increase in the RF. In the sample analysed, the resting RF after transection was around 10 cycle min^{-1} under normal medium, i.e. twice the RF observed in ponto-medullary preparations. No statistically significant age related differences were observed ($9{\cdot}2 \pm 0{\cdot}7$ cycle min^{-1}, $n = 56$, 0–1 day and $10 \pm 1{\cdot}1$ cycle min^{-1}, $n = 39$, 2–4 days).

In six cases where NA (25 μM) increased the RF prior to transection, NA always decreased the RF after transection. In the medullary preparations tested ($n = 62$), NA decreased the RF in fifty-eight cases in a dose-dependent manner (see below), elicited a tonic discharge in thirty cases (Fig. 2*Aa*) and recovery occurred within 6–10 min (Fig. 2*Ab*). No statistically significant age-related differences were observed among the NA responses. When NA (100 μM) was applied, the mean RF was $56{\cdot}5 \pm 10$ and 55 ± 12% of the control RF in rats aged 0–1 days ($n = 7$) and 2–4 days ($n = 14$), respectively. When NA (25 μM) was applied, the mean RF reached 73 ± 11 and 64 ± 10% of the control value in rats aged 0–1 days ($n = 7$) and 2–4 days ($n = 18$), respectively. At weaker concentrations, no age-related differences were observed either.

Noradrenaline agents and respiratory frequency

Table 2 illustrates the dose–response relationship in the case of NA and agonists applied to medullary preparations. Significant decreases in the RF were observed with medium containing NA, adrenaline and phenylephrine. The three drugs were statistically equipotent. The α_2-agonist α-methyl-NA was less potent and elicited significant but more restricted decreases in the RF. The other α_2-agonists (clonidine and guanfacine) and the α_1-agonist (6-fluoro-NA) did not elicit any significant changes in the RF. Figure 2*A* shows one example of the NA-induced decrease in RF and Fig. 2*B–E* the mean changes in RF induced by some of the adrenergic agonists.

The effects of NA were partially or totally blocked by pre-treatment with medium containing the α_2-antagonists yohimbine, piperoxane or idazoxan for 6–9 min. Noradrenaline was less effective after the application of 50 μM-yohimbine and became completely ineffective after 100–200 μM of the antagonist. Figure 3*A* illustrates the average effect of a pre-treatment with the antagonist idazoxan. In other experiments, prazosin, an α_1-antagonist, did not block NA-induced decreases in RF which were statistically even greater than that occurring before the pre-treatment (Fig. 3*B*).

Noradrenaline agents and tonic activation

Noradrenaline (100 μM) elicited a large, continuous discharge on cervical ventral roots in both ponto-medullary (26/50; Fig. 1*B*) and medullary (30/58; Fig. 2*A*) preparations. Lower concentrations of NA (25 μM) were either less effective or ineffective but became effective after pargyline pre-treatment (100 μM; 9 min; $n = 3$)

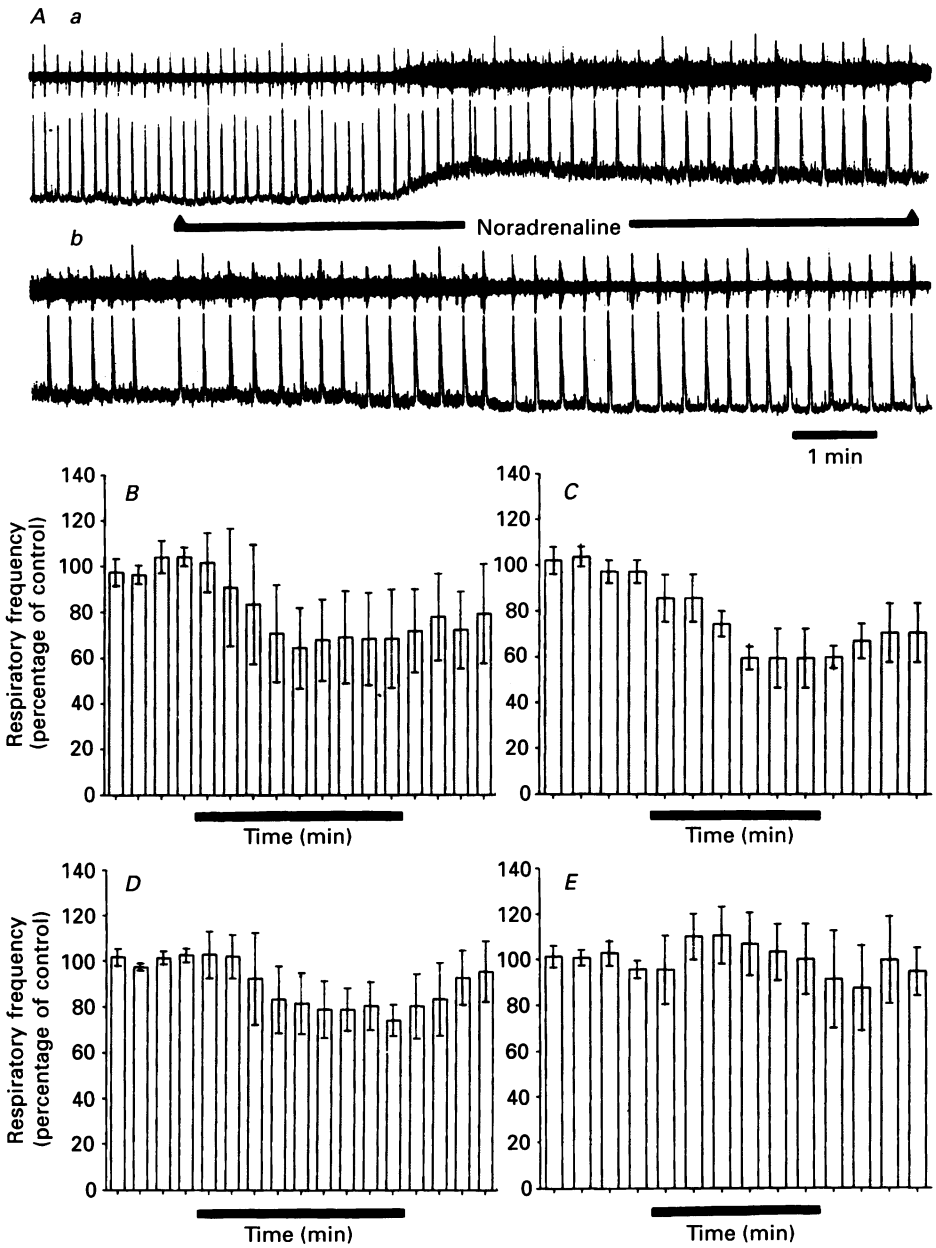

Fig. 2. Effects of noradrenaline (NA) and NA agonists on respiratory activity in medullary preparations. *Aa* and *Ab*, inspiratory activity recorded on a cervical ventral root (upper beam) and integrated activity (lower beam); continuous recording showing the decrease in RF and the tonic discharge elicited when the normal medium was replaced by a medium containing NA (in *Aa*, horizontal bar between arrow-heads) and recovery (in *Ab*). *B–E*, histograms plotted on the basis of several experiments showing the mean effect of NA and some agonists; ordinate, mean RF and S.E.M. expressed each minute as a percentage of the control RF (100%); control RF was the mean RF during a 4 min period under normal medium; abscissa, time in minutes. Standardized protocols used in each

which potentiates the effects of NA by inhibiting the monoamine oxidase (MAO) degradation. The tonic discharge could be modulated with a slow rhythm (1–2 min^{-1}) which was occasionally found to be phase-locked with the respiratory rhythm for a few minutes but the two rhythms were generally independent. As reported above in the case of ponto-medullary preparations, tonic activation was never observed in

TABLE 2. Changes in respiratory frequency induced in medullary preparations

	Concentration in the medium		
	10 μM	25 μM	100 μM
Noradrenaline	-20 ± 15*	-34 ± 9*	-45 ± 13*
(a_1 and α_2)	($n = 8$)	($n = 22$)	($n = 12$)
Adrenaline	-14 ± 12*	-49 ± 20*	-44 ± 8*
(α_1 and α_2)	($n = 4$)	($n = 3$)	($n = 3$)
Phenylephrine	-25 ± 22*	-25 ± 16*	-34 ± 13*
($\alpha_1 > \alpha_2$)	($n = 7$)	($n = 8$)	($n = 7$)
α-Methyl-NA	?	5 ± 8	-18 ± 12*
(α_2)	($n = 0$)	($n = 3$)	($n = 5$)
Guanfacine	?	?	-14 ± 20
(α_2)	($n = 0$)	($n = 0$)	($n = 6$)
Clonidine	-6 ± 11	$+10 \pm 9$	10 ± 15
(α_2)	($n = 8$)	($n = 6$)	($n = 6$)
6-Fluoro-NA	?	?	-15 ± 12
(α_1)	($n = 0$)	($n = 0$)	($n = 10$)
Isoprenaline	?	-1 ± 6	-9 ± 9
(β)	($n = 0$)	($n = 5$)	($n = 7$)

All values are expressed as percentage of control (means ± S.E.M.). Changes induced by replacing the normal medium by media containing different drugs (after 6–9 min of perfusion at the given concentrations). Asterisks indicate statistically significant changes as compared with the control respiratory frequency defined under normal medium before the test.

medullary preparations in the hypoglossal nerve roots, although it was present in simultaneously recorded cervical ventral roots ($n = 8$).

The occurrence of the tonic activity was related to the α_1 potency of the drug added to the bathing medium (see Table 3), since (i) drugs classified as potent selective α_2-agonists (clonidine, guanfacine and α-methyl-NA) or β agonists were almost ineffective and (ii) drugs acting on both α_2 and α_1 sites (NA, adrenaline, phenylephrine) or more selectively on α_1 sites (6-fluoro-NA) frequently elicited a tonic discharge. Furthermore, pre-treatment with the α_1-antagonist prasozin ($n = 9$, 50–100 μM, 6–9 min) prior to NA administration blocked the onset of NA-induced tonic activation. In ten other experiments, NA was tested prior to and after pre-treatment with either the cholinergic antagonist atropine ($n = 5$, 100 μM) or the

experiment: control period at least 4 min; exposure to drug (100 μM), 6 min in *C* and *D* and 9 min in *B* and *D* (horizontal filled bar); then return to normal medium. Note that NA (in *B*, $n = 12$), adrenaline (in *C*, $n = 3$), phenylephrine (in *D*, $n = 7$) but not clonidine (in *E*, $n = 6$) significantly decreased the RF.

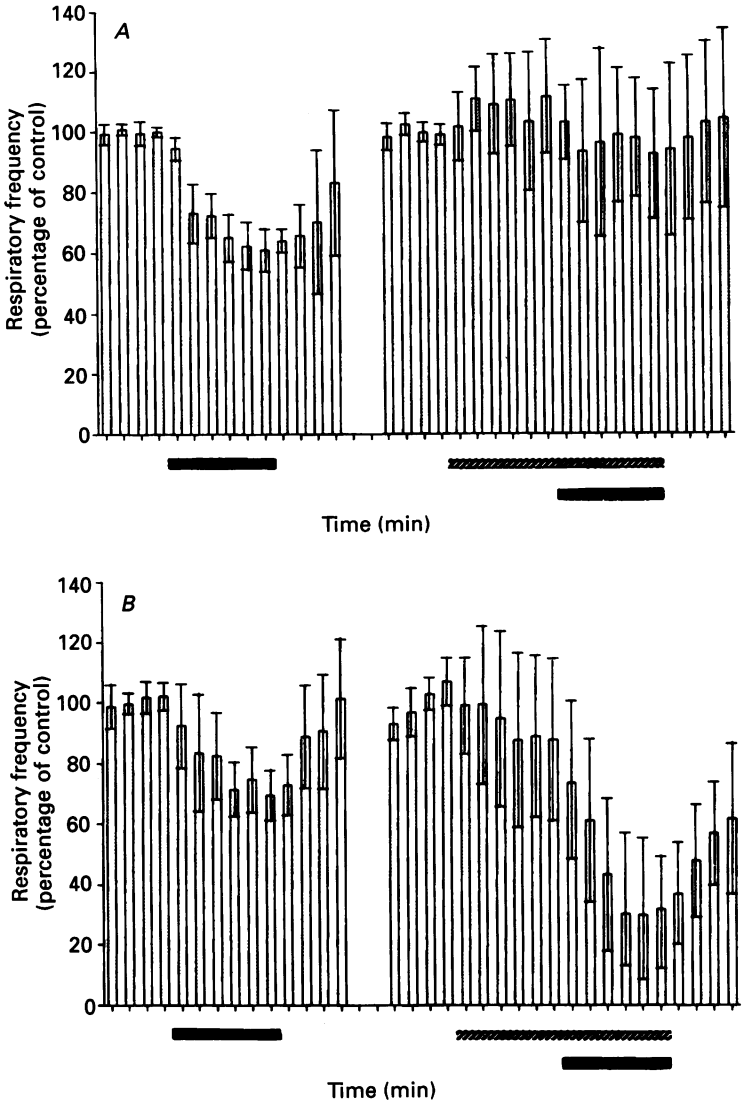

Fig. 3. NA-induced decreases in RF and NA antagonists in medullary preparations. Same arrangement as with the histograms in Fig. 2 showing (in the left part of *A* and *B*) the control histograms when applying NA for 6 min (filled bar), and (in the right part of *A* and *B*) the effects observed 10 min later in the same preparations after pre-treatment with a medium containing the NA antagonist for 6 min (hatched bar) followed by application of NA (filled bar) and the antagonist (hatched bar). *A*, 100 μM-NA ($n = 5$) elicited a significant decrease in the RF under control conditions (on the left); this effect was blocked by pre-treatment with the α_2-antagonist idazoxan (200 μM) (on the right). *B*, 50 μM-NA ($n = 5$) significantly ($-28 \pm 6\%$) decreased the RF (on the left); this effect was potentiated ($-69 \pm 11\%$) by pre-treatment with the α_1-antagonist prazosin (100 μM) (on the right).

serotonergic antagonist methysergide ($n = 5$, 50 μM): NA still decreased the RF but tonic activity was no longer elicited in either case.

In fourteen experiments, the spinal cord was severed from the brain stem at the C1–C2 level. Noradrenaline (100 μM) applied for 9 min to the isolated spinal cord never induced tonic activation. In twelve experiments, a partition was placed at the

TABLE 3. Relationship between the frequency of occurrence (expressed as percentage) of the tonic activity induced by medium containing noradrenergic drugs and type of drug

Drug added to the medium	Tonic activity occurrence (in %)	Selective potency
Noradrenaline	48	α_1 and α_2
Adrenaline	40	α_1 and α_2
Clonidine	0	α_2
α-Methyl-NA	0	α_2
Guanfacine	13	α_2
Phenylephrine	33	$\alpha_1 > \alpha_2$
6-Fluoro-NA	40	α_1
Isoprenaline	0	β_1

C1 level in order to apply NA selectively to the ponto-medullary structures. A tonic activation was elicited in six cases when NA was applied to the pons and medulla (50%) but not in the case of the spinal cord. Microejections of NA performed in the spinal cord (either at the level of the motoneuron pools, -300 μm from the ventral surface, or deeper, -700 μm) never evoked any tonic discharge in the ventral roots.

Sites of action of NA at the medullary levels

With a view to elucidating the mechanisms and the medullary structures involved in the NA respiratory effects, experiments were performed with local application of NA (multibarrelled micropipette and pressure pulses), stimulation and local electrolytic lesions (tungsten microelectrode). In the ventral region of the medulla, two NA-sensitive areas were distinguished: the first, located in the rostro-ventrolateral medulla (RVLM), could be subdivided into two parts (R, rostral and C, caudal) and the second was located in the ventromedial medulla.

The rostral ventrolateral areas

The caudal RVLM. In medullary preparations, ejection of small amounts of NA (1 mM, 5 nl) via a multibarrelled micropipette inserted 200–300 μm below the ventral surface in a well-defined area of the rostral ventrolateral medulla referred to as the C-RVLM (see Fig. 4, filled circle), decreased the RF (Fig. 4*B* and *D*). The mean effect of NA microejections ($n = 25$) was a significant 24% decrease in the RF which occurred immediately after the ejection. Six to eight minutes later, control values were restored. At the same sites, microejections of either saline or clonidine were ineffective: eleven ejections of clonidine had no effect, three increased the RF and three decreased the RF.

At sites where NA decreased the RF, electrical stimulation (1 shock, 300 μs, 50 μA) performed via the central barrel filled with saline triggered inspiration prematurely (Fig. 4*A*). In fourteen additional medullary preparations, electrical stimulations performed with tungsten microelectrodes gave rise to the same effect (Fig. 5*B*). The

inspiratory triggering effect was observed when the stimulation was performed at mid-expiration but never when performed in the very beginning of expiration. Displacing the microelectrode 100 μm in either direction generally led to ineffective points unless the strength of the stimulation was increased (100–200 μA). The

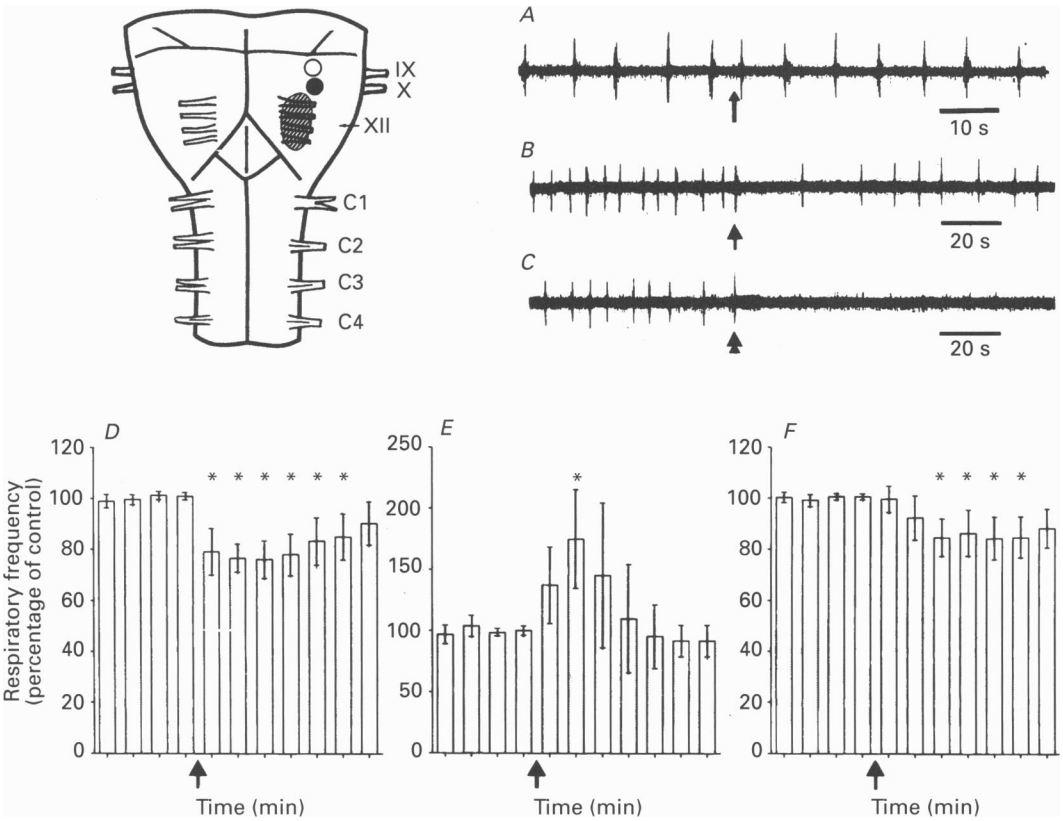

Fig. 4. Medullary sites sensitive to local NA application. On the upper left, schematic drawing showing sites of the medullary ventral surface where NA, locally applied 300 μm below the ventral surface using a multibarrelled micropipette, modified the RF: ○, R-RVLM; ●, C-RVLM; hatched area, ventromedial area. *A–C*, recordings of cervical ventral root activity; a multibarrelled micropipette was inserted 300 μm below the ventral surface at the location of the filled circle; electrical stimulation performed via the central barrel filled with saline triggered inspiration prematurely (*A*, at the arrow), pressure ejection of NA immediately lowered the RF (*B*, at the arrow) whereas saline and clonidine had no effect (not shown) and electrolytic lesion (DC current via the central barrel) stopped the respiratory activity (*C*, at the arrow). *D–F*, same arrangements as in the histograms shown in Fig. 2 illustrating the mean changes in RF elicited every minute by NA ejections (at the arrow) in the filled circle area (C-RVLM in *D*, $n = 20$), the open circle area (R-RVLM in *E*, $n = 7$) and the hatched area (in *F*, $n = 20$). Asterisks indicate significant changes.

efficiency of the single-shock stimulation was not affected by blocking α_1-, α_2- and β-receptors with prazosin, yohimbine and propranolol, respectively (200 μM, 9 min, $n = 5$ with each drug).

At these sites, electrolytic lesions performed with tungsten microelectrode (Fig.

5*C*) or the central barrel of the micropipette (Fig. 4*C*) immediately stopped the respiratory rhythm in all the experiments, but recovery could occur within 3–5 min (Fig. 5*C*). The respiratory activity recovered with a very weak amplitude inspiratory discharge and only half of the control amplitude was restored 10 min after recovery.

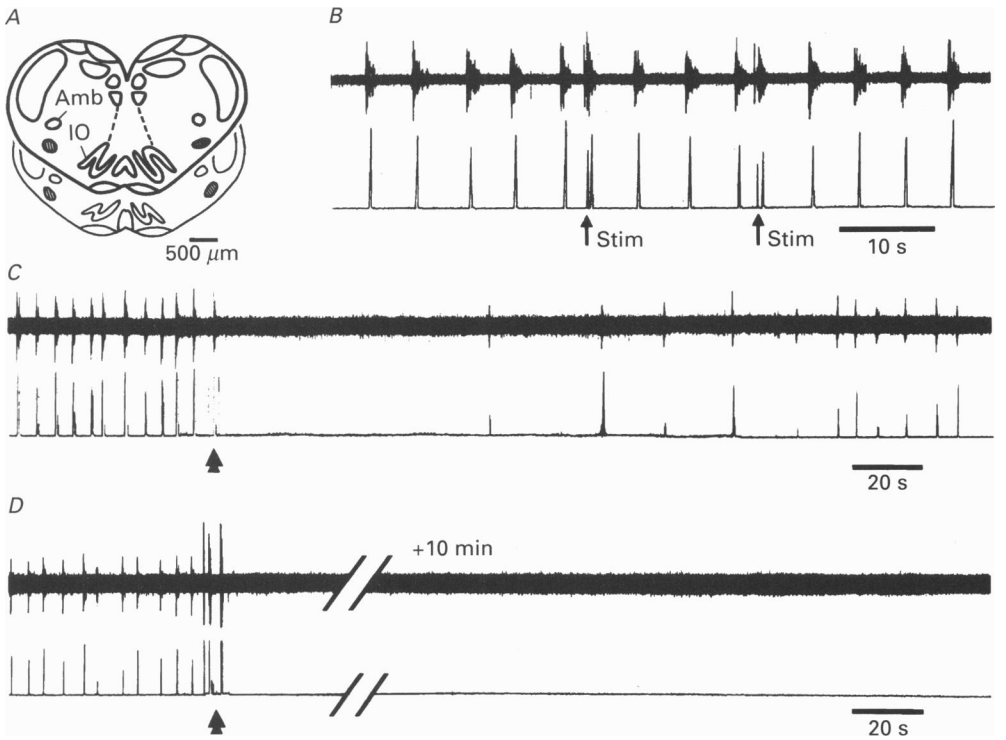

Fig. 5. Effects on respiratory activity of stimulations and lesions in the C-RVLM area in medullary preparations. *A*, schematic drawing from histological slices showing the location of the tungsten microelectrode in the C-RVLM; Amb, nucleus ambiguus; IO, inferior olivary nucleus. Electrolytical lesion areas are indicated by hatching (see filled circle in Fig. 4). *B–D*, raw discharge of a cervical ventral root (upper beam) and integrated activity (lower beam); in a medullary preparation, a tungsten microelectrode was inserted into the hatched area shown in *A*; electrical stimulation performed at the arrow (Stim) triggered inspiration prematurely (*B*); electrolytic lesions in one side stopped respiratory activity temporarily (arrow in *C*). After recovery, a second lesion performed on the contralateral side definitively stopped the respiratory activity (arrow in *D*). The recording was stopped and recommenced 10 min later.

The symmetrical contralateral site was then located using single-shock stimulation (50–100 μA) and lesion was performed with DC current. The second lesion definitively abolished the respiratory activity in 6/9 preparations (Fig. 5*D*). In the three others, respiratory activity was either conserved ($n = 1$) or recovered within 10–15 min ($n = 2$). In these three cases, NA (100 μM) applied after bilateral lesions no longer decreased the RF but tonic activity was evoked in one case.

Histological controls performed in four cases showed that the lesions were located laterally 1·4–1·7 mm from the mid-line, from the ventral surface to 350 μm deeper, below the nucleus ambiguus which was never included in the damaged area. In the

most rostral slice where the lesion area could be seen, the inferior olivary nuclei could still be observed and the VIIth motor nuclei, located more rostrally, were not yet visible (see Fig. 5*A*).

In five ponto-medullary preparations, electrical stimulation of the C-RVLM sites evoked the same inspiratory triggering effects. Electrocoagulation on both sides led

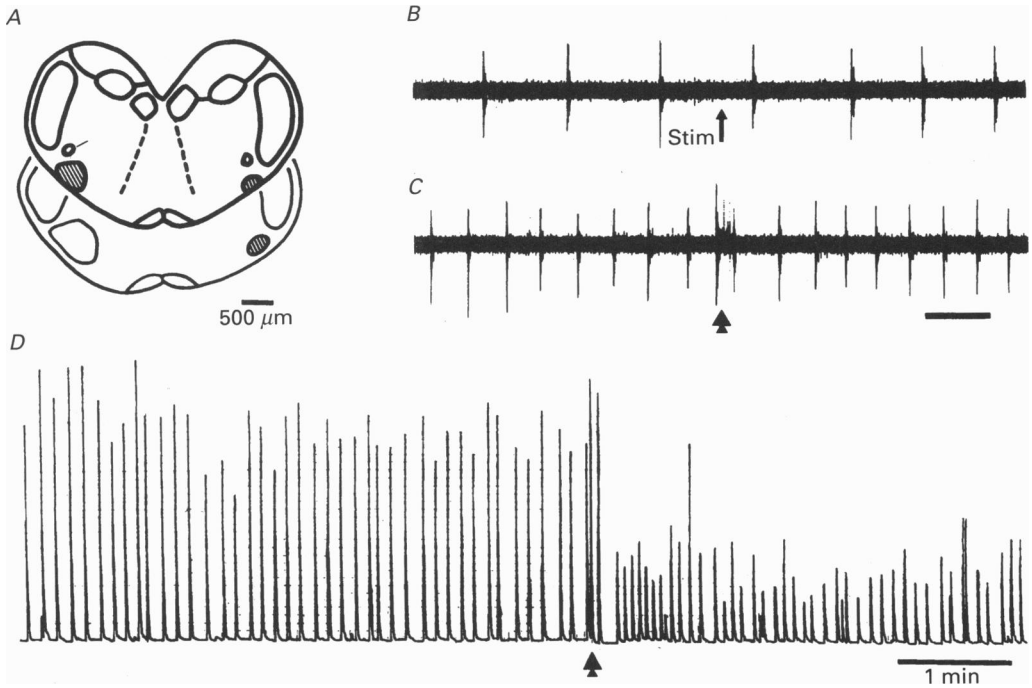

Fig. 6. Effects on respiratory activity of stimulations and lesions in the R-RVLM area. *A–C*, medullary preparation, same arrangement as in Fig. 5 but integrated activity is not shown. The electrode was located in the R-RVLM (hatched area in *A* and open circle in Fig. 4) and neither stimulation (arrow in *B*) nor lesions (arrow in *C*) affected the RF. Time, 5 s in *B* and 10 s in *C*. *D*, ponto-medullary preparation, integrated activity of a cervical ventral root; electrolytic lesion (arrow in *D*) performed in the R-RVLM immediately increased the RF and lowered the amplitude of the integrated inspiratory discharge.

to transient respiratory arrest in 2/5 experiments (1–3 min), and in all the experiments it led to an increase (40–50 %) in the resting RF with a drastic decrease in the amplitude of the respiratory signal, which made it necessary to stop the experiment before NA could be applied.

The rostral RVLM. In five medullary preparations, NA ejected in the most rostral part of the RVLM, referred to as the R-RVLM (Fig. 4, open circle), 300 μm below the ventral surface, increased the RF; saline ejections were ineffective (clonidine was not tested). The mean effect (Fig. 4*E*) reached the statistical significance level 2 min after ejection (75 % of increase) but the magnitude of individual effects was highly variable from one experiment to another, leading to a large S.E.M. (38 %).

When tungsten microelectrode and single-shock stimulation were used, no inspiratory triggering could be obtained (Fig. 6*B*) unless large intensities were used

(> 250 μA, 0·3 ms). Bilateral lesions at the corresponding sites ($n = 7$) did not affect the resting RF (Fig. 6*C*). Noradrenaline applied after limited bilateral lesions (150 μA, 3 s) of these rostral areas still decreased the RF ($n = 5/5$) and elicited tonic activity ($n = 2/5$). Histological controls ($n = 4$) revealed that the lesion areas were

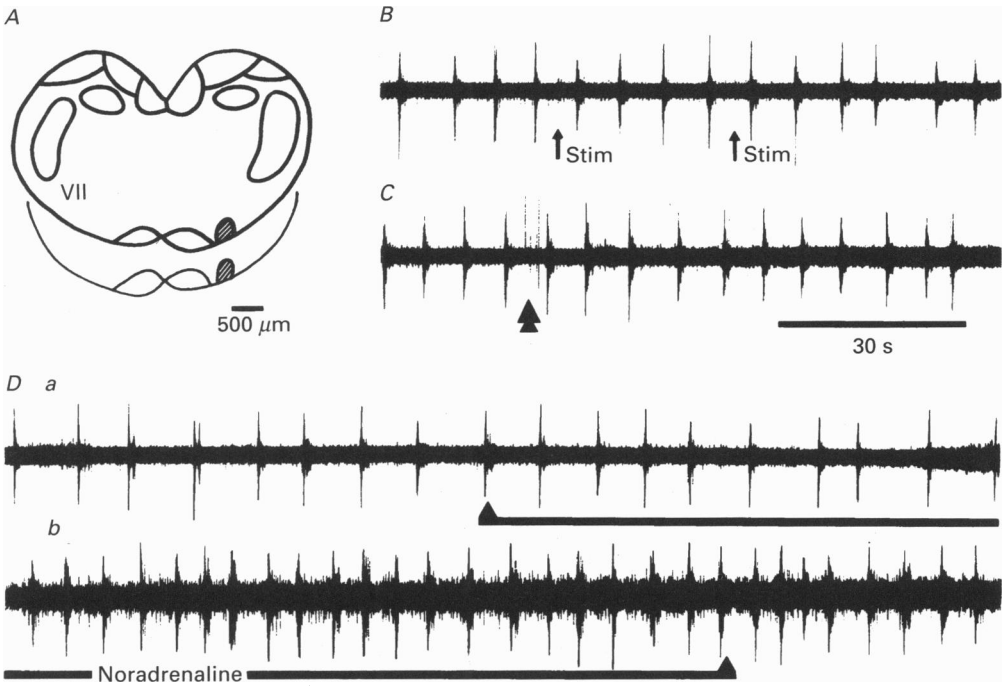

Fig. 7. Effects on respiratory activity and NA bathing response of stimulation and lesions in the ventromedial medulla. *A–C*, same arrangement as in Fig. 6. The electrode was located in the ventromedial medulla (hatched area in *A*, and in Fig. 4) and neither stimulation (arrow in *B*) nor lesion (arrow in *C*) affected the RF in medullary preparations. *Da* and *Db*, after lesions in the ventromedial medulla, bathing of the medullary preparation with a medium containing NA (100 μM) (horizontal filled bar between arrowheads) increased the RF and elicited a tonic discharge in the cervical ventral root (continuous recording).

clearly located rostrally to the C-RVLM. The lesion areas were visible on slices where the inferior olivary nucleus was no longer present. In the most rostral slices, the motor nucleus of the VIIth nerve began to be visible (Fig. 6*A*).

In six ponto-medullary preparations, lesions performed in these rostral areas did not stop the respiratory activity but immediately increased the RF (mean increase, 40%; Fig. 6*D*).

The ventromedial area

In a diffuse area of the ventromedial medulla (Fig. 4; hatched area), extending all along the exit points of the XII roots, sites were encountered where NA ejections often (10/14) decreased the RF. The effects were weaker here than in the C-RVLM, reached the significance level after a longer latency (3 min, see Fig. 4*F*) and lasted

no more than 4 min. In these medial sites, stimulations and bilateral lesions performed via a tungsten electrode 200–300 μm below the ventral surface did not significantly affect the resting RF (Fig. 7*B–C*). After bilateral lesions, however, NA bathing no longer decreased the RF; on the contrary, increases in the RF were observed in 7/16 experiments with a tonic activity in two cases (Fig. 7*D*). The RF changes occurred after a 2 min latency and the mean effect was a 76% increase as compared with the control value, but with a large S.E.M. ($\pm$58%) since both limited (20–40%, $n = 5$) and large (100–200%, $n = 2$) increases occurred. The histological controls did not reveal any special anatomical information about the location (see Fig. 7*A*).

Other tested regions

In fifteen experiments, lesions were performed in other medullary sites without affecting either the respiratory activity or the NA response. A weak decrease in the RF was occasionally observed in several sites after local application of NA, but the reproducibility from one experiment to another was not good enough for these effects to be statistically significant. These sites corresponded to the dorsal medulla (decrease in RF: 2/15 cases in the vicinity of the NTS) and the ventrocaudal medulla 300 μm below the ventral surface (1/7 cases) or deeper (700 μm, 2/14 cases).

DISCUSSION

The results presented here show that NA applied to the medulla of newborn rats (i) elicited tonic discharges on cervical ventral roots and (ii) decreased the respiratory frequency (RF). These changes were due to the activation of medullary α_1- and α_2-receptors, and involved complex mechanisms that have only been partly elucidated. It has been postulated that the decrease in the RF elicited by applying exogenous NA may mimic the modulation of the respiratory rhythm generator by endogenous NA released by A5 (Hilaire *et al.* 1989; Errchidi *et al.* 1990). These results are in agreement with the previous ones obtained *in vivo* where noradrenaline was found to have a depressant effect on respiratory activity (Bolme & Fuxe, 1973; Champagnat, Denavit-Saubie, Henry & Leviel, 1979; Eldridge & Millhorn, 1981). The changes in RF elicited by NA raise some questions concerning both the structures involved and the maturity of the adrenoceptors at birth: the fact that drugs classified as potent α_2-agonists in the cardiovascular field (clonidine and guanfacine) did not decrease the RF as did NA suggests that either the adrenoceptors may be immature at birth or the α_2 classification may need to be revised.

Tonic activity elicited by NA

General application of NA elicited a delayed (2–3 min) tonic activity in cervical roots in half of the experiments. This activation was due to the activation of α_1-receptors since it was blocked by α_1- (but not α_2-) antagonists and was elicited by α_1- (but not α_2-) agonists. These receptors are located in the medulla since tonic activity still occurred after elimination of the pons and could be elicited by NA applied to the medulla but not to the spinal cord. The medullary structures are likely to be quite deep, judging from the 2–3 min latency (diffusion time), but it is not yet possible to

locate them more precisely: NA local application never elicited any tonic discharge and medullary lesions did not suppress the occurrence of NA-induced tonic discharges at any particular site. Similar discharges were elicited in this preparation by both acetylcholine (and blocked by atropine; Monteau, Morin & Hilaire, 1990*c*) and serotonin (and blocked by methysergide; Morin, Hennequin, Monteau & Hilaire, 1990*a*). It has been demonstrated that 5-HT-induced tonic discharge is of spinal origin (Monteau, Morin, Hennequin & Hilaire, 1990*b*; Morin *et al.* 1990*a*). Since both atropine and methysergide blocked the NA-induced tonic activity, cholinergic and serotoninergic circuitry may supply the NA-induced tonic activity. Its functional significance remains to be elucidated, however, although it was reported that in the case of 5-HT, the tonic discharge mainly involved non-respiratory motoneurons which had become tonically active rather than spinal respiratory motoneurons (Morin, Monteau & Hilaire, 1991). The tonic activation could hardly be related to apneustic breathing since (i) the existence of apneusis is doubtful in newborn and adult rats (Monteau, Errchidi, Gauthier, Hilaire & Rega, 1989; Monteau, Gauthier, Rega & Hilaire, 1990*a*) and (ii) the tonic discharge (induced by NA, acetylcholine or 5-HT) is restricted to the cervical roots and never appears in the hypoglossal nerve. Since locomotor activities have been reported to be conserved in this preparation (Smith & Feldman, 1987), it is possible that the tonic discharge may have a locomotor significance.

Noradrenaline medullary sites responsible for the modulation of respiratory frequency

A depressant effect of NA on respiratory activity has already been reported both *in vivo* in the adult animal (for review, see Eldridge & Millhorn, 1981) and *in vitro* in the brain stem preparation of newborn rats (Hilaire *et al.* 1989; Errchidi *et al.* 1990). This depressant effect may reflect the permanent inhibition of the medullary respiratory rhythm generator exerted by A5 catecholaminergic neurons (Hilaire *et al.* 1989; Errchidi *et al.* 1990). In the present study, the decreases in the RF elicited by exogenous NA applied to the medulla are in agreement with these previous reports and may result from the mimetic action of NA on the A5 medullary targets. The increases in the RF elicited by NA in ponto-medullary preparations during double-bath experiments are thought to result from the inhibition of A5 neurons. The inhibition of these neurons by NA (Andrade & Aghajanian, 1982) leads to the withdrawal of the inhibition on the medullary rhythm generator by A5, and therefore to RF increases (Hilaire *et al.* 1989). In the R-RVLM, the increase in RF elicited by NA application may be related either to some medullary sites which mediate NA-excitatory effects on the respiratory rhythm generator or to the most caudal extension of the pontine A5 area (extrapolated from the atlas of the adult brain stem by Paxinos & Watson, 1986). This explanation for the increases in the RF induced by local NA application or by lesions in the R-RVLM agrees with previous work (Hilaire *et al.* 1989).

The NA microejection, lesion and stimulation experiments did not make it possible to definitively locate the medullary A5 targets responsible for NA respiratory modulation, but they made it possible to rule out the involvement of some structures. Ionophoretic application of NA predominantly depressed activity of respiratory cells in adult cats (Champagnat *et al.* 1979) but microejection of NA

700 μm below the ventral surface in sites where the ventral respiratory group of neurons is mainly located in newborn rats (Hilaire, Monteau, Gauthier, Rega & Morin, 1990; Smith, Greer, Liu & Feldman, 1990) did not significantly decrease the RF. The possibility that the decreases in the RF may have been due to a direct NA action on the neurons of the ventral respiratory group can therefore be ruled out. The involvement of the dorsal medulla, specially the dorsal respiratory group, also appears to be unlikely: local lesions or NA applications at dorsal sites had no respiratory effects (present results and Kessler & Jean, 1987) and furthermore, the existence of a dorsal respiratory group in the newborn rat appears to be highly questionable (Hilaire *et al.* 1990).

As revealed by stimulations (which triggered inspiration prematurely) and lesions (which suppressed rhythmic respiratory activity), the C-RVLM may be close to (or may contain) elements of the respiratory rhythm generator. At the same location, NA applications significantly decreased the RF with a very short latency. The effect of NA bathing after bilateral lesions of the C-RVLM (which generally stopped respiratory activity) could only be tested in three instances, but in these three cases, NA no longer decreased the RF. Since (i) elimination of A5 increased the RF (Hilaire *et al.* 1989), (ii) A5 neurons send their axons towards the ventrolateral reticular formation of the medulla (Byrum & Guyennet, 1987) and (iii) lesions in (or rostral to) the C-RVLM increased the RF in ponto-medullary preparations, it can be postulated that these lesions interrupted A5 axons running towards the C-RVLM. Taken as a whole, these results therefore suggest that some neurons in the C-RVLM area play a leading role in respiratory rhythm genesis and may constitute the medullary A5 targets involved in NA modulation of the respiratory rhythm generator.

To attribute the results reported above to a specific cellular type is difficult, if not impossible, since the RVLM is not a homogeneous aggregate of neurons and contains at least three main groups of neurons, all of which are plausible candidates: the C1 noradrenergic neurons, the sympathoexcitatory vasomotor neurons and the neurons of the rostral extension of the respiratory centres. Furthermore, some neurons may be involved in the respiratory changes due to stimulation and lesion and others in the modulation due to NA.

The C1 neurons (Hökfelt, Johansson & Goldstein, 1984) are NA-sensitive neurons which may be involved in cardiovascular regulation (Reis, Granata, Joh, Ross, Ruggiero & Park, 1984) and might mediate the cardiovascular responses initiated from the ventral surface (Benarroch, Granata, Ruggiero, Park & Reis, 1986). Their NA sensitivity seems to argue in favour of the idea that they may be involved in mediating NA modulation to the respiratory rhythm generator. Since the inspiratory triggering evoked by stimulation of the C-RVLM persisted after the adrenoceptor block, adrenoceptors and C1 neurons were not involved in the latter effect. In this area, non-adrenergic cells are intermingled with C1 neurons (Hökfelt *et al.* 1984) and many participate in cardiovascular regulation (Morrison, Milner & Reis, 1988). Some of these cells may exhibit pacemaker properties (Sun, Hackett & Guyenet, 1988*a*; Sun, Young, Hackett & Guyenet, 1988*b*) and constitute a group of sympathoexcitatory cells firing with a respiratory modulation (Haselton & Guyenet, 1989). Cardiovascular and respiratory neurons are also intermingled in the rostro-ventrolateral medulla and constitute two separate but widely overlapping areas

(Ellenberger & Feldman, 1990; Ellenberger, Feldman & Zhan, 1990). This site appears to be a critical link between the central respiratory network and the sympathetic vasomotor outflow (Guyenet, Darnall & Riley, 1990). Under our *in vitro* conditions, where the peripheral loops were eliminated, central interactions between cardiovascular and respiratory networks may still take place at both the A5 and RVLM levels.

A particular type of respiratory neuron has been reported to play a 'trigger' role in respiratory rhythmicity in this *in vitro* preparation (Onimaru & Homma, 1987; Onimaru, Arata & Homma, 1987; Arata, Onimaru & Homma, 1990). Since these neurons seem to be widely distributed in the rostro-caudal direction (see Fig. 2*B e–h* from Arata *et al.* 1990), their extension does not fit the localized effects of stimulation and lesion reported here. In adult cats, comparable inspiratory triggering effects were reported after stimulation of an area located ventrolaterally to the facial nucleus, where respiratory neurons sending axons to all the respiratory groups were also assumed to play a critical role in respiratory rhythmicity (Smith, Morrison, Ellenberger, Otto & Feldman, 1989; Connelly, Ellenberger & Feldman, 1990). In the *in vitro* preparation, stimulations and lesions in the vicinity of the facial nucleus had no respiratory effects and therefore did not support this assumption. In the newborn rat, a detailed mapping of the respiratory centres has been published recently (Smith *et al.* 1990), which confirms that existence of some respiratory neurons in the C-RVLM area, at a depth of 300 μm from the ventral surface. The critical zone leading to inspiratory triggering and arrest may be the most rostral and ventral part of the respiratory column (see Fig. 13 from Smith *et al.* 1990), where a particular group of inspiratory neurons is located in a limited area caudal to the facial nucleus and very ventral. This group might correspond to the ventrolateral subgroup of the Bötzinger complex in the adult rat, which contains loosely packed respiratory propriospinal and bulbospinal neurons (Ellenberger & Feldman, 1990; Ellenberger *et al.* 1990). This subgroup may be responsible for the stimulation and lesion results we have described, and possibly also for the effects of NA, although NA may act indirectly via non-respiratory neurons of the RVLM.

The C- and R-RVLM areas are unlikely, however, to be the only structures mediating NA effects, since local NA application in some sites of the ventromedial medulla also affected the RF. From the long latency and the weak magnitude of the decrease in the RF, these areas seem unlikely to play a leading role, but lesions in these sites reversed the effects of NA. This suggests that at these sites, some structures may relay the C-RVLM control or that the medial sites may define the NA sensitivity of both the C- and R-RVLM. As α_1-receptor block by prazosin potentiated the depressant effects of NA (see Fig. 3*B*), the possibility cannot be excluded that the RF changes due to NA bathing may have resulted from a dual effect; an inhibition mediated by the α_2-receptors in the C-RVLM and a facilitation mediated by α_1-adrenoceptors in the R-RVLM. After medial lesions, the excitatory role of the R-RVLM might be exacerbated. It is difficult to attribute to any anatomically or functionally known structure the medial sites distributed diffusely in the medulla. They might be linked to the medial subgroup of the C1 area (see Paxinos & Watson, 1986) in agreement with the response to locally applied NA. They might also correspond to the chemosensitive areas which extend close to the ventral surface,

rostro-caudally over the whole medulla, and which are involved in central respiratory activity in both adult (for review, see Millhorn & Eldridge, 1986) and newborn animals (Monteau *et al.* 1990*c*). The RF response to NA was not affected, however, by atropine which modifies chemosensitivity (Monteau *et al.* 1990*c*), and it therefore seems unlikely that the chemosensitive areas may have been involved.

To conclude, some neurons of the C-RVLM may be the medullary A5 targets which participate in mediating A5 noradrenergic modulation on the respiratory rhythm generator but it is not possible at present to define more precisely the type of neurons and the complex mechanisms involved in this modulation.

Types of adrenoceptors involved in the modulation of respiratory frequency

The results reported here show that the RF was decreased in response to NA, adrenaline, α-methyl-NA and phenylephrine. The endogenous catecholamines, NA and adrenaline, activate both α_1- and α_2-adrenoceptors whereas α-methyl-NA is a selective α_2-agonist and phenylephrine acts on α_1- and to a lesser degree on α_2-receptors (Cedarbaum & Aghajanian, 1977; Andrade & Aghajanian, 1982). Since the depressive effect of NA on the medullary respiratory rhythm generator was blocked by α_2- (yohimbine, piperoxane, idazoxan) but not α_1- (prazosin) antagonists, and was not evoked by specific α_1- (6-fluoro-NA, Chiueh *et al.* 1983) and β-agonists (isoprenaline), it seems quite plausible that α_2-receptors have been involved. The RF did not decrease, however, in response to the α_2-agonists clonidine (Cedarbaum & Aghajanian, 1977) and guanfacine (Jarrot *et al.* 1982) and decreased only weakly with α-methyl-NA. The discrepancy between the results obtained with agonists and antagonist makes it difficult to draw any definitive conclusions as to either the agonist potency and specificity of these agents or the maturity of the receptors involved.

Specificity of α_2-agonists

Clonidine was the most extensively analysed α_2-agonist; this antihypertensive agent has been classified as a potent and selective α_2-agonist on the basis of biochemical and pharmacological data (U'Prichard, 1981). When injected into the nucleus reticularis lateralis, however, clonidine evoked hypotension but the α_2-agonist α-methyl-NA did not (Bousquet, Feldman, Bloch & Schwartz, 1981), and some results have brought its α_2 specificity into question (Bousquet & Feldman, 1986). α-Methyl-NA is catecholaminergic in structure but clonidine is an imidazoline. It was therefore first suggested that 'there may be some form of structure–activity relationship which would indicate the existence ... of sites preferring the imidazoline structure' (Bousquet, Feldman & Schwartz, 1984). Finally, binding and physiological experiments led to the conclusion that the clonidine vasodepressor response is mediated by imidazol but not α_2-receptors (Ernsberger, Giuliano, Willette & Reis, 1990).

On the one hand, clonidine has α_2-agonist effects at the pontine level since it inhibited A5 neurons when applied iontophoretically (Andrade & Aghajanian, 1982) and elicited increases in the RF when injected into A5 (Hilaire *et al.* 1989) or applied to the pons (present study). On the other hand, clonidine has no α_2-agonist potency when applied to the medulla or injected into the RVLM. During *in vivo* experiments, clonidine was reported to cause a 15–20% decrease in RF (Bolme & Fuxe, 1973;

Bolme, Corrodi, Fuxe, Hökfelt, Lidbrink & Goldstein, 1974) but peripheral and indirect effects may have been involved and it is therefore impossible to draw any definitive conclusions about the actual central respiratory effects of clonidine.

Maturity of adrenoceptors at birth

Another explanation for the absence of NA-mimetic effects of clonidine and guanfacine is the possible immaturity (in terms of number, distribution, structure, responsiveness or combination of all the above) of the medullary α_2-receptors at birth. Developmentally different onsets of α_1- and α_2-adrenergic responses have been reported for neurons in the dorsal motor nucleus of the vagus (Fukuda, Nabekura, Ito, Plata-Salaman & Oomura, 1989). Our results show that the use of antagonists points to the existence of differences between α_1- and α_2-responses (NA-induced decreases in RF are blocked by α_2- but not α_1-antagonists) but that some α_2-agonists were not efficient. Furthermore, clonidine modified the RF when applied to the pons but not to the medulla; this suggests that the maturation of adrenoceptors occurs earlier at the pontine than at the medullary level. The onset of adrenergic responses may therefore differ depending on both the type (α_1/α_2) and the location (pons/medulla) of the receptors. Within the period we analysed (0–4 days), no differences were observed in the NA-induced decrease in RF between 0- to 1- and 2- to 4-day-old rats, suggesting that the number and efficiency of the α_2-receptors involved in respiratory control did not change during the first 4 days after birth. Changes may occur, however, during the following days of life. If so, this raises the question as to what neurovegetative changes may occur at the onset of the maturation of the α_1/α_2 receptors. This could be physiologically significant, as NA modulates various functions (digestive, cardiovascular and respiratory). Moreover, the NA neurons which innervate the raphe nuclei may modify the synthesis and release of 5-HT, which has been reported to modulate RF and the drive to the upper airways (Monteau *et al.* 1990*b*; Morin *et al.* 1990*a*, *b*). Any dysfunction in NA receptor maturation might therefore have multiple consequences which might be parallel to the various symptoms reported in cases of Sudden Infant Death Syndrome. It is worth mentioning that abnormal catecholamine synthesizing enzyme activity has been reported in brain stem areas in victims of Sudden Infant Death Syndrome (Denoroy, Gay, Gilly, Tayot, Pasquier & Kopp, 1987) and the abnormal urinary catecholamine levels have been detected in pre-term infants with apnoea (Kattwinkel, Macs, Farnoff & Klaus, 1976).

Still in connection with the maturation of central respiratory control, the significance of the A5 modulation may be questioned. It should be noted that the resting RF in ponto-medullary preparations was slower in 0- to 1-day-old rats than in 2- to 4-day-old rats; this may reflect changes in the modulation of the respiratory rhythm generator exerted by A5. No differences were observed in the resting RF of medullary preparations, which suggests that the medullary networks operate identically at 0–1 and 2–4 days of age. If neither the medullary respiratory networks nor the α_2-adrenoceptor responses are affected by age between 0 and 4 days, the differences in the RF in ponto-medullary preparations can only have been due to some pontine structure affecting the RF, probably A5. Just after birth, the mean RF is slower than at 2–4 days of age and this may reflect the extinction with age of an

inhibitory control which may have been powerful before birth. On similar lines, the incidence of fetal breathing was almost abolished by NA infused intravenously in chronic fetal rhesus monkey (Murata, Martin, Miyake, Socol & Druzin, 1981). The possibility that A5 may modulate the respiratory rhythm generator has not yet been confirmed in adult animals; this inhibitory modulation may be responsible for the periods of non-respiratory activity commonly described in the fetus (for review, see Dawes, 1984; Moss & Inman, 1989).

To conclude, the results of the present experiments performed in the *in vitro* brain stem preparation of newborn rats suggest that the activity of the medullary respiratory rhythm generator is modulated by the A5 neurons via α-adrenoceptors (probably α_2) located in the RVLM. This inhibitory modulation might be connected with the apneic periods occurring during fetal life. The possible immaturity of the adrenoceptors at birth and the magnitude of the A5 modulation remain open questions in view of the drastic consequences which might result from a sudden maturation of these receptors during the first days of life.

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