5-HT_{2A/2C} Receptor–Mediated Hypopnea in the Newborn Rat: Relationship to Fos Immunoreactivity

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ABSTRACT

Previous data derived from anesthetized, decerebrate, or *in vitro* preparations suggested that 5-HT₂ receptor activation might be responsible for respiratory dysfunction. Such a mechanism has not yet been documented in the intact animal, but might be of clinical relevance to the apneic spells of the premature infant. In the present investigation on conscious newborn rats we analyzed the respiratory response to the activation of 5-HT_{2A/2C} receptors by the agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), and we delineated central structures possibly involved in this response, using Fos expression as a marker of neuronal activation. We demonstrated that intraperitoneal injection of 5 mg/kg DOI produced a long-lasting decrease in respiratory frequency and tidal volume, which could be blocked by the antagonist ritanserin. Fos immunohistochemistry suggested that the rostral ventrolateral medulla and the lateral para-

gigantocellular nucleus might have a key role in the respiratory response to 5-HT₂ receptor activation. In addition, double immunostaining for Fos and tyrosine hydroxylase suggested that the contribution of catecholaminergic neurons to this response might be modest and indirect. (*Pediatr Res* 50: 596–603, 2001)

Abbreviations

DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane

FLI, Fos-like immunoreactive

NTSc, commissural subdivision of the nucleus of the solitary tract

AP, area postrema

RVL, rostroventrolateral medulla

LPGi, lateral paragigantocellular nucleus

GiA, gigantocellular reticular nucleus, alpha

Serotonin (5-hydroxytryptamine; 5-HT) acting at 5-HT₂ receptors has been involved in the neuromodulation of a broad spectrum of functions ranging from mental processing to autonomic regulation (1). 5-HT also interferes with breathing control, *via* central and peripheral 5-HT₂ receptors, and variable responses have been reported. *In vitro* studies on brain stem–spinal cord preparations isolated from the newborn rat indicated that central 5-HT_{2A} receptor mechanisms may exert facilitating influences on respiration, by activating spinal respiratory motoneurons (2) and increasing the frequency of rhythmic respiratory discharge (3). In contrast, in adult anesthetized rats, the activation of 5-HT₂ receptors located at the periphery has been involved in decreases in phrenic nerve activity and lung compliance observed during apnea induced by 5-HT (4). In decerebrate newborn rats, the application of 5-HT_{2A/2C} receptor agonists to the floor of the fourth ventricle elicited a bradypnea (5), reminiscent of the decrease in frequency of respiratory-like activity that followed bath applica-

tion of 5-HT_{2C} receptor agonists to *in vitro* preparations (3). Furthermore, the finding that 5-HT_{2A/2C} receptor agonists inhibit hypoglossal inspiratory activity in isolated brain stem–spinal cord preparations led to the hypothesis that 5-HT₂ receptors may be involved in the genesis of obstructive apneas in newborns with increased 5-HT biosynthesis (2, 6). Nevertheless, the depressant influence of 5-HT₂ receptor activation on hypoglossal activity has not been confirmed on medullary rhythmic slice (7). Altogether, those findings suggest that 5-HT₂ receptor mechanisms might influence the network devoted to respiratory control at multiple levels and that the response to 5-HT₂ receptor activation in the intact animal may not be definitely deduced from those in simplified or anesthetized preparations. In particular, evidence that 5-HT₂ receptor activation may lead to respiratory disturbances in the conscious newborn is still missing, although it might be of clinical importance as regards recurrent apneic spells in the premature infant. Indeed, the likelihood of 5-HT₂ receptors being activated increases with the concentration of serotonin synthesized or released centrally. This concentration has been reported to be abnormally elevated in neonates with intrauterine growth retardation (8) and notably during hypoxia (9). Furthermore, although serotonin acting at 5-HT₂ receptors might be benefi-

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cial to adults suffering from pathologic apneas in that it decreases the duration of deep slow-wave sleep (10), this mechanism might be inefficient in the neonate (11).

The present investigation was aimed at a better understanding of the contribution of 5-HT_{2A/C} activation to the regulation of breathing, using conscious newborn rats. We analyzed the respiratory responses to systemic administrations of DOI, or/and ritanserin, both acting on 5-HT_{2A/C} receptors as 5-HT agonist and antagonist, respectively.

Fos immunohistochemistry is now commonly accepted as a tool to map polysynaptic neuronal pathways (12). It was used in the present investigation to delineate subsets of medullary neurons that might mediate the respiratory response to 5-HT_{2A/C} receptor activation, by mapping neurons activated after DOI administration, preceded or not by a blockade of 5-HT_{2A/C} receptors by ritanserin.

Finally, as changes in the ratio of catecholamine to 5-HT release may contribute to respiratory disturbances (7), double-immunohistochemical labeling directed to Fos and tyrosine hydroxylase was used to determine the response of catecholaminergic neurons to 5-HT₂ receptor activation.

MATERIALS AND METHODS

The experimental protocols were carried out in conformity with the European Communities Council Directive (86/609/EEC) on 82 newborn Sprague-Dawley rats (0–3 d).

DOI (RBI) was given at doses of 2.5, 5 and 8 mg/kg. Ritanserin (Sigma/RBI, Natick, MA, U.S.A.) was given at a single dose of 0.4 mg/kg. These doses were selected on the basis of existing published information (13). DOI was dissolved in normal saline and ritanserin in 0.4% acetic acid in saline, and the pH was adjusted to 7.4 with sodium hydroxide. Both drug solutions were prepared immediately before use and injected intraperitoneally as a single 0.05-mL bolus.

In each tested group, ventilatory variables and Fos expression were studied in separate subsets of six to eight newborn rats. The following treatments were delivered: DOI, ritanserin, or vehicle solution in a single injection (control 1); pretreatment with ritanserin followed 40 min later by DOI injection or two injections of vehicle solution (saline followed by 0.4% acetic acid in saline) spaced by a 40-min interval (control 2).

Animals used for recording ventilatory variables were transferred to a 60-mL experimental chamber in which they could freely move. The chamber was continuously ventilated with humidified air and maintained in the thermoneutral zone (32°C). It was connected to a differential pressure transducer (Validyne DP45, Validyne Engineering Corporation, Northridge, CA, U.S.A.; sensitivity: ± 2 cm H₂O) to record the pressure changes induced by the respiratory flow, using a modification of the plethysmographic technique described by Bartlett and Tenney (14). Signals were digitized through a Spike2 (CED, Cambridge, U.K.) data analysis system. Frequency and tidal volume were evaluated at fixed intervals of 5 min, on a minimum of 10 respiratory cycles, and for a maximum of 2 h, to avoid any stress-related effect of starvation or dehydration. The first measurements were done during the 15 min preceding either saline or drug injections and were used as

control values. Further changes in ventilatory parameters were expressed in percentages, relative to the baseline control values (100%). At the end of the experiments, rats were either killed or were returned to the dam for checking their ability to recover from treatment. Neither single injections of DOI or ritanserin nor the combination of both drugs impaired newborn development or survival.

Animals used for Fos immunohistochemistry were returned to the dam immediately after injection and killed 2 or 3 h after the last injection by an overdose of pentobarbital (100 mg/kg) containing heparin (250 IU/kg). They were transcardially perfused with 0.1% xylocain in saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain stems were removed, postfixed overnight, and stored in cryoprotectant until freeze sectioning.

An additional group of newborn rats was used to check the influence of 5-HT₂ receptor activation on body temperature. Rats were separated from their dam, weighed and injected intraperitoneally with a single 0.05 mL bolus of saline ($n = 3$) or of DOI ($n = 3$; 5 mg/kg). Each animal was identifiable from a specific mark drawn on its back with indian ink. After injection, pups were pooled together into a single cage placed under a heating lamp. Their rectal temperature was measured for 2 h, at fixed 10 min intervals, using a small diameter probe lubricated with liquid paraffin and connected to an electronic thermocouple. The heating lamp was moved up or down to analyze the effects of DOI under conditions of hypo-, normo-, and hyperthermia.

Fos immunohistochemistry was conducted on serial 40 μ m-thick transverse sections of the brain stem as routinely done in the laboratory (15). Briefly, every second section was incubated for 48 h in a rabbit polyclonal antibody raised against Fos synthetic peptide (Santa Cruz Biochemicals, Santa Cruz, CA, U.S.A., diluted 1/4,000). Fos-like immunoreactivity was detected by the avidin-biotin technique using commercial kits (Vectastain Elite, Vector Laboratories, Peterborough, UK) containing a biotinylated goat anti-rabbit IgG and an avidine-biotinylated peroxidase complex (ABC). Peroxidase activity was revealed by 0.02% diaminobenzidine (DAB) in the presence of 0.01% H₂O₂ and enhanced by 0.04% nickel ammonium sulfate.

Separate sets of sections were processed for double immunostaining of Fos and tyrosine hydroxylase (TH). They were incubated in a mixture of the Fos antibody and a polyclonal antibody against native tyrosine hydroxylase raised in sheep (Chemicon International, Temecula, CA, U.S.A.; diluted 1/4,000) for 48 h at 4°C. Both immunoreactivities were detected using the ABC method. Fos was revealed first, as described above. Then, TH was revealed using a biotinylated secondary antibody raised in rabbit and directed against sheep IgG. After the ABC procedure, peroxidase was revealed by DAB (0.05%), without nickel intensification. Under these staining conditions, the nuclei of Fos positive neurons appeared in black and the cytoplasm of TH immunoreactive neurons appeared in brown under the light microscope.

In every animal, all of immunostained sections were examined under light microscopy. Anatomical localization and regional nomenclature were defined according to Paxinos (16)

with the exception of the paraolivary and parapyramidal groups at the ventral medullary surface (17). Accordingly, TH immunoreactive neurons were included in the A1, A1/C1, or C1 groups when they were identified in the ventrolateral medulla caudal to the aperture of the fourth ventricle, at the level of the pyramidal decussation (A1) or rostral to it (A1/C1), and at the level of the fourth ventricle (C1), respectively. The A5 group encompassed neurons distributed lateral to the facial nucleus. TH immunoreactive neurons were included in the A2 or C2 groups when they were identified in the dorsomedial medulla caudal or rostral to the aperture of the fourth ventricle, respectively.

The distribution of FLI neurons was plotted onto drawings performed with the aid of a drawing tube attached to the microscope (objective $\times 4$ or $\times 10$). Simple and double-labeled neurons were counted at higher magnification (objective $\times 20$) using an eyepiece fitted with a squared grid to avoid neurons to be counted more than once. Counts were performed in individual anatomical structures along their entire longitudinal extent. Individual values were expressed as mean numbers of cells per structure and per section, on one side of the brain stem. Group averages of the numbers of labeled neurons per section and of the consecutive sections of separate structures in which labeling was present were determined. They were presented together with SEM (Table 1).

Data on respiratory and Fos responses to treatment were analyzed by Wilcoxon signed rank test (Statview+ package, SAS Institute, Cary, NC, U.S.A.) and were considered significant at $p < 0.05$.

RESULTS

Respiratory response to 5-HT_{2A/2C} receptor activation. Rat pups injected with saline ($n = 6$) responded to handling by a transient increase in respiratory frequency from 133 ± 15 to 163 ± 6 breaths/min within the first 15 min of measurements. They gradually recovered within the following 20 min (Figs. 1 and 2A). The effects of DOI depended on the dose delivered. DOI injected at 5 mg/kg rapidly elicited a marked reduction in ventilation ($n = 6$). Within 20 min after drug administration, respiratory frequency and tidal volume decreased up to $56 \pm 4\%$ and $66 \pm 2\%$ of pretreatment value, respectively. Respiratory frequency and tidal volume reached a plateau and remained below control values although DOI-injected rats did not significantly differ from controls at the end of the run of recording (Figs. 1 and 2A). No apneic spell could be recorded.

At 2.5 mg/kg ($n = 3$), DOI did not significantly affect either tidal volume or respiratory frequency (Fig. 3). On the other hand, animals treated with 8 mg/kg ($n = 3$) showed major behavioral effects, preventing any reliable measurement of ventilation.

Although DOI elicited marked changes in the breathing pattern, it did not alter rectal temperature whether the injected rat pups were hypothermic (DOI *versus* control value: $35.2 \pm 0.5^\circ\text{C}$ *versus* $35.2 \pm 0.1^\circ\text{C}$), normothermic ($38.1 \pm 0.2^\circ\text{C}$ *versus* $38.1 \pm 0.1^\circ\text{C}$), or hyperthermic ($41.1 \pm 0.2^\circ\text{C}$ *versus* $41.0 \pm 0.2^\circ\text{C}$).

By itself, the antagonism of 5-HT_{2A/2C} receptors by ritanserin ($n = 6$) had no effect on respiratory frequency (Fig. 2B). In

Table 1. Fos expression in the medulla and the pons of control and drug-injected newborn rats (mean numbers of FLI neurons per section $\pm$ SEM)

	Control 1 $n = 8$	DOI $n = 8$	Ritanserin $n = 6$	Control 2 $n = 8$	Ritanserin + DOI $n = 8$
NTSc	75.7 ± 19.7	$140.8 \pm 27.8^*$	24.5 ± 10.3	105.8 ± 33.8	125.6 ± 33.1
<i>n</i>	4.0 ± 0.6	6.2 ± 0.5	4.5 ± 0.9	5.8 ± 0.6	6.6 ± 0.5
AP	20.8 ± 8.1	$53.0 \pm 12.9^*$	10.7 ± 9.1	53.0 ± 19.0	88.8 ± 28.7
<i>n</i>	3.6 ± 0.7	5.3 ± 0.6	4.5 ± 0.5	3.5 ± 0.5	4.1 ± 0.4
RVL	19.6 ± 5.5	$36.9 \pm 8.2^*$	$0.6 \pm 0.3^*$	19.5 ± 7.0	16.8 ± 5.0
<i>n</i>	5.6 ± 1.8	4.8 ± 1.2	4.2 ± 1.2	5.1 ± 0.7	5.6 ± 0.9
Facial nucleus	32.8 ± 6.4	45.4 ± 5.0	19.8 ± 3.3	35.4 ± 4.0	25.9 ± 4.0
<i>n</i>	7.1 ± 0.6	8.1 ± 0.7	6.4 ± 1.0	6.8 ± 0.9	7.1 ± 0.6
LPGi (juxtaf)	4.1 ± 1.5	$15.4 \pm 2.8^*$	1.5 ± 0.9	2.9 ± 1.0	5.9 ± 1.8
<i>n</i>	3.6 ± 0.8	4.8 ± 1.1	3.5 ± 1.2	5.8 ± 1.4	6.1 ± 1.2
GIA	10.7 ± 1.9	$18.3 \pm 3.0^*$	5.3 ± 1.9	15.0 ± 4.2	19.8 ± 3.9
<i>n</i>	5.8 ± 0.6	7.2 ± 0.6	5.0 ± 1.3	6.4 ± 0.8	7.8 ± 1.2
R Pal	12.5 ± 1.3	11.5 ± 1.3	$5.4 \pm 1.3^*$	12.1 ± 2.8	$23.3 \pm 3.7^*$
<i>n</i>	17.1 ± 1.1	19.8 ± 1.2	15.7 ± 1.2	15.1 ± 1.3	15.6 ± 1.2
R Mg	9.3 ± 1.5	9.1 ± 1.2	4.6 ± 1.5	9.2 ± 2.4	$14.4 \pm 2.5^*$
<i>n</i>	8.2 ± 1.4	8.0 ± 1.4	7.5 ± 1.1	6.0 ± 0.7	7.3 ± 1.2
R Ob	12.9 ± 3.0	10.4 ± 2.3	7.7 ± 3.0	15.1 ± 4.8	$31.8 \pm 5.4^*$
<i>n</i>	9.8 ± 0.9	10.5 ± 0.9	8.2 ± 1.1	9.3 ± 0.8	10.3 ± 0.8
PO	5.2 ± 0.7	6.3 ± 2.2	$0.9 \pm 0.4^*$	3.9 ± 0.8	$7.6 \pm 1.2^*$
<i>n</i>	7.5 ± 1.1	9.1 ± 1.0	7.3 ± 0.9	8.1 ± 0.8	10.3 ± 0.7
PP	8.5 ± 2.0	7.7 ± 1.7	$0.3 \pm 0.2^*$	9.1 ± 2.4	$25.3 \pm 6.1^*$
<i>n</i>	7.2 ± 0.9	6.8 ± 0.6	6.5 ± 1.1	5.3 ± 1.4	7.0 ± 0.9
PB	14.9 ± 3.4	9.8 ± 1.9	17.2 ± 3.6	19.3 ± 4.9	20.2 ± 2.9
<i>n</i>	4.4 ± 1.3	4.5 ± 2.0	4.0 ± 0.5	4.2 ± 0.7	4.0 ± 0.5

* Indicates significant difference between control and stimulated animals.

n, mean numbers ($\pm$ SEM) of sections containing FLI neurons per animal; RPal, ROb, RMag, nu. raphe pallidus, obscurus and magnus; PO, paraolivary group; PP, parapyramidal group; PB, parabrachial complex.

Figure 1. Plethysmographic recordings from newborn rats following injection of DOI (5 mg/kg) or saline (control). Vertical bar: mean predrug amplitude of the pressure signal.

contrast, it blocked the response to 5-HT_{2A/2C} receptor activation within a 20-min delay after injecting 5 mg/kg DOI ($n = 6$) (Fig. 2C).

The administration of ritanserin elicited a moderate but sustained increase in tidal volume (Fig. 2B). The difference between ritanserin- and vehicle-injected rats was significant 25, 40, and 45 min after injection. At this time, tidal volume

peaked at 138% of predrug value. The pretreatment with ritanserin fully prevented the depressant effect of DOI on tidal volume (Fig. 2, B and C).

FLI evoked by 5-HT_{2A/2C} receptor activation. The pattern of Fos expression has been examined in rat pups treated with 5 mg/kg DOI, compared with saline-injected animals. The present analysis focused on the medullary and pontine areas devoted to respiratory and autonomic control. Two hours after a single DOI injection, the number of FLI neurons was significantly above control values in several medullary regions ($n = 8$) (Table 1). These included the NTSc ($p < 0.035$), the AP ($p < 0.020$) (Fig. 4B and Fig. 5a), the RVL ($p < 0.020$, the LPGi ($p < 0.042$) (Fig. 4B, and Fig. 5b), and the GiA ($p < 0.025$) (Fig. 4B and Fig. 5c). DOI did not significantly alter immunostaining in the intermediate (DOI versus control: 65.4 ± 10.4 versus 41.1 ± 9.7 neurons/section) or rostral portions of the NTS (2 ± 1.1 versus 4.3 ± 1.2), and in the nucleus ambiguus (4.4 ± 1.8 versus 3.7 ± 1.6). The pattern of Fos expression was very similar in the rat pups killed 3 h after treatment ($n = 8$), except from a decrease in the number of immunostained neurons in the LPGi (2 h versus 3 h, 15.4 ± 4.8 versus 6.4 ± 1.4 neurons/section). Thus, the effects of ritan-

Figure 2. Time course of the changes in respiratory frequency (Rf) (A1, B1, C1) and tidal volume (VT) (A2, B2, C2) elicited by 5-HT_{2A/2C} receptor manipulation. Variations are expressed as mean percentages of predrug value $\pm$ SEM. (A1, A2) Effects of saline (control) and 5 mg/kg DOI injected at time 0. (B1, B2) Effects of saline (control) and 0.4 mg/kg ritanserin injected at time 0. (C1, C2) Effects of saline and DOI injected at time 0 after pretreatment with ritanserin 40 min before. Asterisk (*) marks a significant difference between control and drug-injected group.

Figure 3. Average changes in respiratory frequency (*Rf*), expressed as mean percentages of predrug value $\pm$ SEM, elicited by 2.5 and 5 mg/kg DOI at different times after injection.

serin alone or ritanserin plus DOI had further been evaluated on rats killed 2 h after the last drug injection.

Compared with control values, an overall decrease in Fos expression was observed after the injection of 0.4 mg/kg ritanserin (Table 1). This decrease was significant in the RVL ($p < 0.027$), the raphe pallidus nucleus ($p < 0.015$) and in the paraolivary ($p < 0.027$) and parapyramidal areas ($p < 0.006$). This suggests that, in these areas, ritanserin might have attenuated the effects of handling and/or of injection on Fos expression.

After sequential administration of ritanserin and DOI, Fos expression remained at control values in the RVL and the LPGi. This indicated that the activation of 5-HT₂ receptors highly contributed to Fos expression evoked by DOI in these structures.

In the NTSc, the AP, and the GiA, the numbers of FLI neurons after DOI injection were very similar whatever the animals were pretreated or not with ritanserin. Nevertheless, the difference between vehicle- and drug-treated rats did not reach the level of significance, mostly because increased basal Fos expression after repeated injections.

In addition, pretreatment with ritanserin appeared to unmask a significant Fos response to DOI in the raphe nuclei ($0.027 < p < 0.046$) and in the paraolivary ($p < 0.027$) and parapyramidal groups ($p < 0.027$) (Table 1).

DOI-induced Fos expression in catecholaminergic neurons. Double Fos/TH-positive neurons were intermingled with single Fos- and TH-positive neurons in the pontine and medullary catecholaminergic groups (Fig. 6). After DOI administration, the number of double-labeled neurons per section was moderate but significantly above control values in A1 (5.4 ± 1.8 versus 0.3 ± 0.1 ; $p < 0.043$), A1/C1 (6.3 ± 1.0 versus 1.9 ± 1.0 ; $p < 0.027$), A2 (4.4 ± 0.9 versus 1.3 ± 0.6 ; $p < 0.043$), C2 (2.6 ± 0.7 versus 0.6 ± 0.1 ; $p < 0.046$) and A5 (2.1 ± 0.4 versus 0.7 ± 0.2 ; $p < 0.025$). Neurons in the locus ceruleus were strongly immunoreactive for TH, but none was positive for Fos.

Figure 4. Schematic drawings of transverse sections showing the distribution of Fos-positive neurons (black dots) in the medulla of newborn rats. A1–A3: control; B1–B3: DOI injection. Amb, ambiguus nucleus; CVL, caudoventrolateral medulla; IO, inferior olive; MVe, medial vestibular nucleus; NTSi, intermediate subnuclei of the nucleus of the solitary tract; PrH, prepositus hypoglossal nucleus; Rmg, raphe magnus nucleus; SpVe, spinal vestibular nucleus; 5sp, spinal trigeminal nucleus; 7, facial nucleus; 12, hypoglossal nucleus.

The proportions of double-labeled neurons, expressed as percentages of TH or Fos immunoreactive neurons, are presented in Table 2. After DOI injection, the fraction of TH neurons expressing Fos ranged from 25% to <40% in the different catecholaminergic groups of the medulla. The double-labeled neurons accounted for approximately 15–26% of the total number of Fos-positive cells in the ventral groups (A1, A1/C1, A5) and only 3–4% in the dorsal ones (A2, C2). The difference between treated and control rats was the most marked in the A1 region. On the whole, our data indicated a moderate contribution of catecholaminergic neurons to DOI-induced Fos expression in the medulla and the pons of newborn rats and that these neurons were mostly distributed ventrally.

Figure 5. Photomicrographs of transverse sections of the brain stem showing Fos-positive neurons after DOI injection in (a) the NTSc and AP, (b) the RVL, and (c) the LPGi and GiA at the level of the facial nucleus (7). Scale bar: 0.5 mm.

DISCUSSION

This investigation demonstrated that the activation of 5-HT_{2A/2C} receptors elicits a long-lasting respiratory depression in the conscious newborn rat that could be blocked by prior antagonism, and that this response was mirrored in the pattern of Fos expression in the RVL and the LPGi.

From previous investigations on various experimental models, the activation of serotonergic pathways may have opposite effects on ventilation and on respiratory neuron activity, depending on the site of application and on the targeted receptor or neuronal subtypes (18). We focused on the effects of the activation of 5-HT₂ receptors, triggered by systemic injections of DOI and ritanserin. Both cross the blood-brain

Figure 6. Photomicrographs of the A1/C1 region from a DOI-injected newborn rat showing neurons immunoreactive for Fos (↓) and tyrosine hydroxylase (star) together with double-labeled neurons (↓↓). Scale bar: 0.05 mm.

barrier and have similar affinities for the 5-HT_{2A} and 5-HT_{2C} subtypes (1). Hence, the respiratory depression we observed in the conscious newborn rat was likely to represent the net effect of the concomitant activation of peripheral and central 5-HT_{2A/2C} receptors.

A decrease in breathing rate has been observed after administration of DOI to the fourth ventricle in the decerebrate rat pup (5). It is reminiscent of the decrease in respiratory-like burst frequency elicited by the application of m-CPP, a selective 2-HT_{2C} agonist, to brain stem–spinal cord preparations (3). Hence, the influence of DOI on breathing rate *in vivo* suggests that central 5-HT_{2C} receptor mechanisms dominate in the unanesthetized newborn. As indirect support to this hypothesis, the central distribution of 5-HT_{2C} receptors is much more extensive than that of 5-HT_{2A} receptors (19).

Together with a decrease in breathing rate, DOI elicited a reduction in tidal volume. Both an increase in airway resistance and a depression of “pump” muscle activity could explain this response. The former hypothesis is supported by earlier findings that 5-HT_{2A/2C} receptor activation induced bronchoconstriction in the adult rat (4) and depressed hypoglossal activity *in vitro* in brain stem preparations from neonatal rats (2). In contrast, spinal motoneurons were excited by 5-HT_{2A/2C} receptor agonists, which suggested that 5-HT_{2A/2C} receptor activation might rather contribute to increase tidal volume in the intact animal by reinforcing chest muscle activity (2). The present observation that DOI induced a decrease in tidal volume raised the issue whether increase in airway resistance may prevail over chest muscle activation in the intact newborn rat.

Whereas the activation of 5-HT_{2A/2C} receptors by DOI elicited a decrease in breathing rate and tidal volume, their blockade by ritanserin increased tidal volume without modifying respiratory rate. This suggests that endogenously released 5-HT might exert a tonic moderating control on tidal volume

Table 2. Colocalization of Fos and TH (mean percentage values $\pm$ SEM)

	%TH-Fos/TH		%TH-Fos/Fos	
	Control	DOI	Control	DOI
A1	1.3 $\pm$ 0.6	36.5 $\pm$ 9.2*	1.9 $\pm$ 0.5	25.8 $\pm$ 4.1*
A1/C1	15.3 $\pm$ 5.5	28.7 $\pm$ 6.7	5.3 $\pm$ 2.7	14.6 $\pm$ 3.1*
A5	11.9 $\pm$ 5.9	24.9 $\pm$ 7.3	6.7 $\pm$ 1.4	20.7 $\pm$ 3.6*
A2	10.9 $\pm$ 6.7	37.4 $\pm$ 9.6*	1.8 $\pm$ 0.9	3.0 $\pm$ 0.9
C2	15.9 $\pm$ 8.1	28.8 $\pm$ 10.1	2.4 $\pm$ 0.9	4.0 $\pm$ 1.2

* Indicates significant difference between control and stimulated animals.

via 5-HT_{2A/2C} receptor mechanisms. On the other hand, 5-HT_{2A/2C} receptors seem not to contribute to the tonic control of the respiratory rhythm generator in the intact animal, in eupneic conditions. Thus, they apparently differ from 5-HT₁ receptors that exert a tonic facilitating action on respiratory rhythmogenesis (3). A possibility is that circulating and/or central serotonin levels normally reach the threshold for activating 5-HT₁ but not 5-HT₂ receptor mechanisms involved in the control of the respiratory rhythm generator, consistent with the lower affinity of the latter subtype for the endogenous ligand (1).

Although both respiratory rate and tidal volume decreased after systemic administration of DOI, the present data did not indicate that serotonin acting on 5-HT_{2A/2C} receptor might be responsible for respiratory arrest in the conscious newborn, in contrast to previous finding in the anesthetized adult rat (4). As reported, the apnea elicited by the 5-HT_{2A/2C} receptor agonist α -methyl-5-HT could be blocked by bilateral vagotomy below the nodose ganglion. Among possible explanations for the difference between the conscious newborn and the anesthetized adult rat are the following: i) peripheral 5-HT_{2A/2C} receptor mechanisms that triggered apnea might be balanced by peripheral and/or central excitatory inputs that are depressed by anesthetic; ii) the vagal pathway triggered by 5-HT_{2A/2C} receptor activation might not be fully mature in the newborn; or iii) the physiologic response to 5-HT_{2A/2C} agonists could depend on the relative occupancy of central and peripheral receptors, which might differ according to the agonist used or the route of administration. Nevertheless, the present data support the idea that 5-HT₂ receptor mechanisms might contribute to respiratory dysfunction under conditions of increased 5-HT levels in the newborn (6). Notably, although apparently not primarily responsible for apnea in a normoxic environment, the activation of 5-HT₂ receptors might have a negative influence on the newborn's ability to recover from sustained apneic episodes when hypoxemia develops. This might increase the vulnerability of small-for-age neonates, which exhibit an inappropriate acceleration of serotonin synthesis during hypoxia (9). Furthermore, independent of the occurrence of hypoxic episodes, 5-HT₂ receptor activation might work together with altered respiratory muscle function (20) to impair ventilation in growth-retarded neonates with increased serotonin synthesis (8).

The blockade of the respiratory response to DOI injection by pretreatment with ritanserin was mirrored in the loss of DOI-induced Fos expression in the RVL and the LPGi. This labeling pattern was consistent with the presence of both 5-HT_{2A} and 5-HT_{2C} in these regions (19, 21). Although neuronal function

cannot definitely be deduced on the sole basis of Fos expression, the present data suggest that the activation of neurons in the RVL and the LPGi might play an active role in the respiratory depression elicited by 5-HT₂ receptor activation. This hypothesis is supported by earlier findings that the application of DOI at the surface of the intermediate area, a region immediately ventral to the RVL and the LPGi elicited a marked decrease in respiratory rate together with a depression of recurrent laryngeal and phrenic nerve activities in the anesthetized cat (22). Alternatively, the possibility should be considered that Fos expression might be partly related to side effects of hypoventilation, such as hypoxia and/or hypercapnia, and/or related cardiovascular adjustments. Nevertheless, the distribution of Fos expression evoked by these stimuli was much more extensive than after DOI injection (15, 23).

Whereas pretreatment with ritanserin blocked the Fos response to DOI in the RVL and the LPGi, the reverse occurred in the medullary raphe nuclei, the paraventricular, and the parapyramidal areas. Anatomical evidence of direct and reciprocal connections between the ventrolateral and the ventral medial medulla has been provided (24). The present data suggest that both regions might negatively interact. Further investigations are needed to determine whether these interactions might be of functional importance to the modulation of the central respiratory drive by serotonergic inputs.

Although the prevention of DOI-induced Fos expression by ritanserin appeared to be a valuable index of the contribution of definite areas to the physiologic response to 5-HT_{2A/2C} receptor activation (13), the reverse may not inevitably be true, due to limitations of the Fos technique (12). This might be the case for the NTS, where ritanserin did not significantly attenuate the Fos response to DOI in the NTS, although it contributes to breathing control and contains 5-HT₂ receptors (19, 21). Possibly, some solitary neurons might not up-regulate *c-fos* at detectable levels, even if strongly activated by 5-HT₂ receptor mechanisms. Furthermore, the effects of ritanserin on the Fos response to DOI might have been blunted, due to the contribution of side events. Indeed, compared with our recent evaluation in conscious rat pups (15), the present control values suggest that a sizeable part of Fos expression in the NTS resulted from handling and/or injection procedures. This response could have partly been mediated by endogenous serotonin acting on 5-HT₂ receptors, because the numbers of Fos positive neurons in the NTS were lower in ritanserin- than in saline-injected rats. In such a situation of extensive occupancy of 5-HT₂ receptors by the endogenous ligand, subsequent binding with ritanserin might have partially been prevented.

The finding that DOI elicited Fos expression in a subset of TH immunoreactive neurons raised the issue of the contribution of catecholaminergic neurons to the respiratory response to 5-HT₂ receptor activation. In particular, according to previous data (25), the activation of noradrenergic neurons in the A5 region might have contributed to the decrease in respiratory frequency we observed. Nevertheless, Fos expression was much more enhanced in TH-positive A5 neurons under conditions of increased ventilation (17, 26). Furthermore, to our knowledge, the presence of 5-HT₂ receptors on A5 neurons has not been reported. Thus, the interactions between 5-HT₂ receptor mechanisms and noradrenergic pathways are likely to be indirect and deserve further investigations to be established.

On the whole, the distribution pattern of the TH/Fos neurons after DOI injection differed from that reported after cardiorespiratory challenges, such as severe hypoxia (12), hypotension, or hypertension (26). The percentage of Fos-positive neurons that also contained TH in the ventral catecholaminergic groups were lower after DOI injection (15–26%) than after hypoxia (28–51%) (17) or hypotension (63–79%) (26). Both stimulations, as well as stress (27), led to dense Fos expression (80–100%) in the locus ceruleus (A6 group), where we did not identify any FLI neuron. Less than 5% of the Fos neurons were also TH positive in the dorsal A2, C2 groups after DOI injection whereas they were up to 40% after hypoxia, hypertension, or hypotension (17, 26). There was virtually no double-labeled neuron in the ventral catecholaminergic groups after hypertension (26). Altogether, those findings provide further arguments against the hypothesis that the respiratory and Fos responses to 5-HT₂ receptor activation may be the indirect consequence of the pharmacological manipulation of baro- or chemoreceptor pathways, or of stress.

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