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Serotonergic modulation of central respiratory activity in the neonatal mouse: an in vitro study

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Abstract

In order to determine whether the serotonergic modulation of the central respiratory activity previously reported in neonatal rats occurs in species other than the rat, we performed identical in vitro experiments on the neonatal mouse to those performed on the neonatal rat. The effects of adding serotonin (5-hydroxytryptamine, 5-HT) and related agents to the superfusate suggested that the respiratory rhythm generator undergoes an excitatory modulation via medullary 5-HT_{1A} receptors. Upon applying the drugs to the spinal cord alone, 5-HT was found to have a dual effect on phrenic motoneuron firing: (i) a facilitatory effect mediated by 5-HT_{2A} receptors and (ii) a depressive effect on their inspiratory discharge mediated by non-5-HT_{1A}, non-5-HT_{2A}, non-5-HT_3 receptors, possibly of the 5-HT_{1B} subtype. It was therefore concluded that serotonin modulates the neonatal central respiratory activity in mice as well as in rats, and that similar 5-HT receptor subtypes are involved in this process in both species. © 1997 Elsevier Science B.V.

Keywords: Respiratory network, central; Neonatal mouse; Serotonergic regulation; 5-HT (5-hydroxytryptamine, serotonin); (In vitro)

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT), a widely distributed neurotransmitter (for review, see Osborne, 1982; Peroutka, 1994), modulates numerous functions in mammals, including the neurovegetative functions and especially the working of the central respiratory network. The authors of in vivo studies have frequently reported the occurrence of respiratory changes resulting from the application of 5-HT agents (Holtman et al., 1986, 1987; Lalley, 1986a,b; Mitchell et al., 1992) but they have often yielded conflicting results due to differences between the modes of drug administration used (i.e. central versus peripheral effects, for example see Millhorn et al., 1983). In vitro experiments carried out on brainstem-spinal cord preparations from neonatal rats (Morin et al., 1990, 1991a,b; Lindsay and Feldman, 1993; Monteau et al., 1994; Di Pasquale et al., 1992, 1994), a method which circumvents the undesirable peripheral effects (Suzue, 1984), have consistently confirmed that 5-HT modulates the activity of the central respiratory network in terms of both its frequency and its amplitude. Briefly, 5-HT has been found (1) to exert an excitatory modulation on the rat respiratory rhythm generator via medullary 5-HT_{1A} receptors (Morin et al., 1990, 1991b; Monteau et al., 1994; Di Pasquale et al., 1992, 1994), (2) to increase the excitability of the phrenic motoneurons via spinal postsynaptic 5-HT_{2A} receptors (Morin et al., 1991a; Lindsay and Feldman, 1993), and (3) to depress the transmission of the central respiratory drive to the phrenic motoneurons via spinal presynaptic non-5-HT_{2A} receptors (Lindsay and Feldman, 1993). The aim of the present study was to determine whether these effects of 5-HT are specific to the neonatal rat respiratory network, or whether they may also take place in species other than the rat. We therefore first adapted to the mouse the in vitro brainstem-spinal cord preparation previously used with the rat, and then repeated on the neonatal mouse some of the experiments previously performed on the neonatal rat. The results obtained lead to the conclusion that 5-HT modulates the activity of the central respiratory network at birth, in mice as well as in rats, and that similar 5-HT receptor subtypes are involved in this process in both species. Since the mouse is extensively used in genetic studies (but not the rat) and mutant mice with 5-HT metabolism abnormalities are now available (Cases et al., 1995), the results of this study mean that it is now possible to perform compar-

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ative in vitro experiments on normal versus mutant mice with a view to understanding more fully the role of 5-HT in the maturation, regulation and working of the central respiratory network.

2. Materials and methods

2.1. Electrophysiological experiments

Experiments were carried out in vitro on 102 medulla-spinal cord preparations from neonatal mice (postnatal days 0–3; strain OF1 from IFFA-CREDO breeding centre). As described elsewhere with rats (Suzue, 1984), the mouse medulla and spinal cord were isolated and superfused with artificial cerebro-spinal fluid (aCSF, in mM: NaCl 129, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.15, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 30.0; pH 7.4, gassed with 95% O_2/5% CO_2, and maintained at 26.5 ± 1°C). Suction electrodes were used to record the activity of the C4 phrenic roots. The signals were filtered (5–3000 Hz), amplified (5 K) and fed to leaky integrator (time constant 50 ms), oscilloscope and paper recorder. In order to be able to make comparisons with the data obtained on rats (Morin et al., 1990, 1991a,b; Lindsay and Feldman, 1993; Monteau et al., 1994; Di Pasquale et al., 1992, 1994), the experimental procedures, pharmacological tools and data compilation methods used here were identical to those previously used with rats.

2.2. Drugs

The following drugs were diluted in aCSF and applied for 4 min (5-HT and 5-HT receptor agonists) or 10 min (other agents): 5-HT (Sigma), (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT, Research Biochemicals International) as 5-HT_{1A} receptor agonist, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl] piperazine hydrobromide (NAN-190, Research Biochemicals International) and 4-iodo-*N*-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-benzamide hydrochloride (p-MPPI, Research Biochemicals International) as 5-HT_{1A} receptor antagonists, 5-methoxy 3(1,2,3,6-tetrahydro 4-pyridinyl) 1-*H*-indol-succinate (RU24969 from Roussel-UCLAF) and 7-trifluoromethyl-4(4-methyl-1-piperazinyl)-pyrrolo[1,2-*a*]quinoxaline, 1:2 maleate (CGS-12066B, Research Biochemicals International) as 5-HT_{1B} receptor agonists, *S*(−)-1-(1*h*-indol-4-yloxy)-3-[(1-methylethyl)amino]-2-propanol ((−)-pindolol, Research Biochemicals International) as 5-HT_{1B} receptor antagonist, 2,5-dimethoxy-4-iodoamphetamine hydrobromide (DOI, Research Biochemicals International) and α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT, Research Biochemicals International) as 5-HT_{2A} receptor agonists, {*trans*, 4-([3*Z*)3-(2-dimethylaminoethyl) oxyimino-3(2-fluorophenyl)propen1-yl]phenol hemifumarate} (SR 46349B, SANOFI-Recherche) as 5-HT_{2A} receptor antagonist and granisetron (Beecham Pharmaceuticals) as 5-HT_3 receptor antagonist. In some experiments, the recording chamber was divided by placing a barrier, improved with vaseline, at the level of the first cervical root in order to apply the aCSF containing a drug to either the medulla or the spinal cord (Fig. 1A).

2.3. Analysis of the results

The number and amplitude of the integrated phrenic discharges were measured every minute during a period of at least 5 min prior to any drug application to estimate the control mean respiratory frequency and the control mean amplitude of the integrated phrenic bursts under normal aCSF. Drugs were then applied and the resulting changes

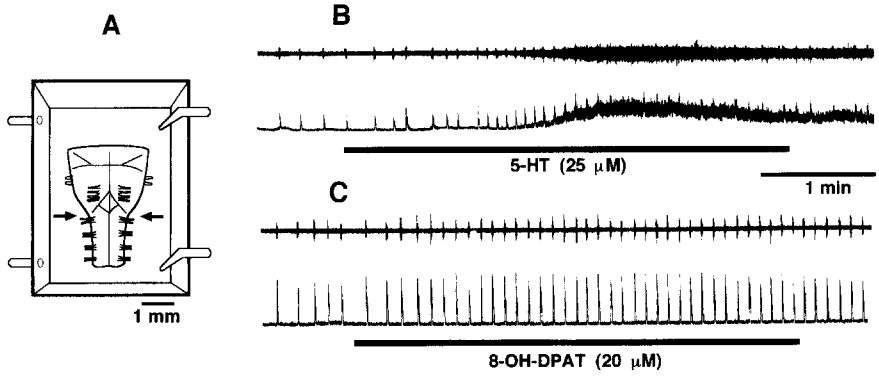

Fig. 1. Changes in central respiratory activity induced by 5-HT in the in vitro medulla-spinal cord preparation from neonatal mice. (A) Schematic drawing of the in vitro medulla-spinal cord preparation from neonatal OF1 mice, with the ventral surface upward; arrows indicate the position of the barrier used for double-bath experiments (see text and Fig. 3). (B) Replacing the normal aCSF superfusing the preparation by aCSF containing 5-HT (25 μM, 4 min) (i) increased the respiratory frequency (estimated from the frequency of occurrence of the phrenic bursts; top and bottom traces, raw and integrated phrenic discharges, respectively), (ii) induced a phrenic tonic discharge and (iii) decreased the amplitude of the inspiratory phrenic bursts. (C) Applying the 5-HT_{1A} receptor agonist 8-OH-DPAT (20 μM, 4 min) instead of 5-HT (i) increased the respiratory frequency, (ii) did not elicit a tonic discharge in the phrenic root and (iii) did not depress the amplitude of the phrenic bursts.

were expressed as % of the control values. The experiments were repeated on several preparations with a standardized procedure to evaluate any differences (means ± S.E.M.), which were taken to be significant at $P < 0.05$ (Student's t-test for paired and unpaired comparisons).

3. Results

As in the neonatal rat, the central respiratory network of the neonatal mouse continues to produce rhythmic bursts in the phrenic roots in vitro with little variability in either the frequency or the amplitude. A coefficient of cycle duration variability was defined as the S.D./mean ratio recorded during 60 successive respiratory cycles. The individual coefficients calculated ranged from 0.09 to 0.31 (mean value ± S.E.M., 0.20 ± 0.07, $n = 10$). The mean resting respiratory frequency and the mean duration of the inspiratory bursts were 5.4 ± 0.3 min^{-1} and 890 ± 30 ms, respectively ($n = 30$). Although survival of the preparations could exceed 5–6 h with no striking changes in the rhythmic phrenic activity, the following pharmacological experiments were performed during the first hour of recording. As shown in Fig. 1B, replacing the normal aCSF by aCSF containing 5-HT (25 μM) gave rise to 3 reversible effects: (i) an increase in the respiratory frequency, (ii) the occurrence of a tonic discharge in the phrenic nerve and (iii) a decrease in the amplitude of the phrenic bursts.

3.1. Respiratory frequency and serotonin

In 14 preparations, applying aCSF containing 5-HT (25 μM) for 4 min increased the respiratory frequency from 5.3 ± 0.9 min^{-1} (normal aCSF) to 9.8 ± 1.3 min^{-1} in 2–3 min (Fig. 2A). The normal values were recovered within 4–5 min of resuming the normal aCSF. Four different concentrations of 5-HT were applied (6, 12, 25 and 50 μM with $n = 4$, 8, 14 and 4 mice, respectively). At each of these concentrations except 6 μM, the mean respiratory frequency calculated taking the 4-min application of 5-HT increased significantly in a dose-dependent manner (107 ± 10, 155 ± 28, 169 ± 23 and 193 ± 21% of the control values, 100%, Fig. 2B). Applying 5-HT to the medulla alone (25 μM) and normal aCSF to the spinal cord increased the respiratory frequency (139 ± 21%, $n = 4$) while applying 5-HT to the spinal cord alone and normal aCSF to the medulla had no significant effect on the respiratory frequency. The respiratory frequency increased significantly upon applying the 5-HT_{1A} receptor agonist (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT; 20 μM, 134 ± 12%, $n = 6$, Fig. 1C and Fig. 2C) but not the 5-HT_{2A} receptor agonists (Fig. 2C) 2,5-dimethoxy-4-iodoamphetamine hydrobromide (DOI; 30 μM, 107 ± 11%, $n = 6$) and α-methyl-5-HT (30 μM, 108 ± 9%, $n = 6$). The increases in the respiratory frequency

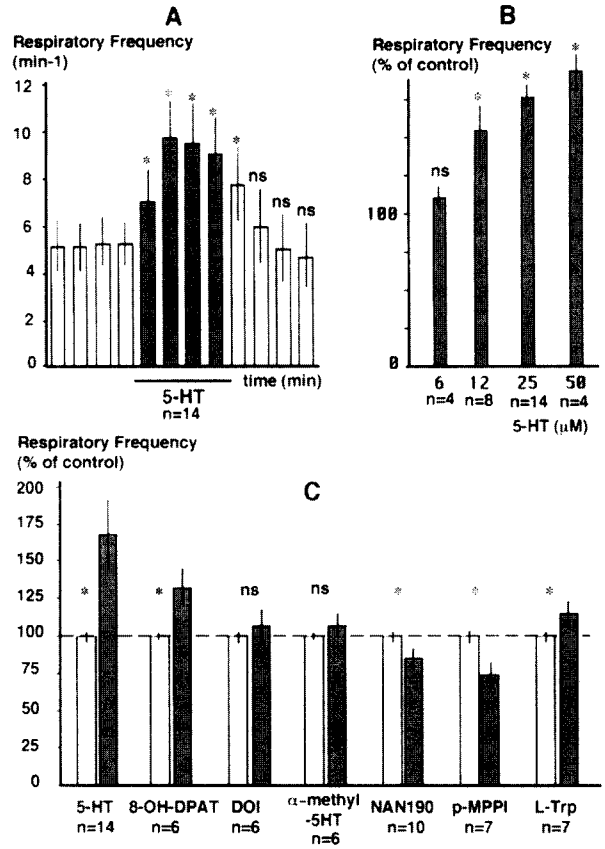

Fig. 2. Modulation of respiratory frequency by 5-HT. Asterisks and ns indicate significant and non-significant changes, respectively ($P < 0.05$). (A) Columns and bars show the mean respiratory frequency and S.E.M. (expressed in min^{-1}, $n = 14$) calculated every minute versus time (in minutes) when the normal aCSF (white columns) was replaced for 4 min by aCSF containing 5-HT (25 μM, grey columns). (B) Columns and bars show the mean respiratory frequency and S.E.M. (RF, calculated during 4-min application of 5-HT and expressed as a percentage of the control values, 100% = value prior 5-HT application) versus 4 different concentrations of 5-HT in aCSF. (C) Columns and bars show mean respiratory frequency and S.E.M. (expressed as a percentage of the control) under normal aCSF (white columns) and aCSF containing drug (grey columns); 5-HT (25 μM), the precursor L-tryptophan (L-Trp, 50 μM) and the 5-HT_{1A} receptor agonist 8-OH-DPAT (20 μM) significantly increased the respiratory frequency; the 5-HT_{2A} receptor agonists DOI (30 μM) and α-methyl-5-HT (30 μM) had no significant effect; the 5-HT_{1A} receptor antagonists NAN190 (40 μM) and p-MPPI (40 μM) significantly depressed the respiratory frequency.

induced by aCSF containing 5-HT (25 μM) could be prevented by applying a 10-min pre-treatment with aCSF containing the 5-HT_{1A} receptor antagonists NAN190 (40 μM, $n = 3$) and p-MPPI (40 μM, $n = 7$) but not the 5-HT_{2A} receptor antagonist SR46349B (20 μM, $n = 5$). Applying aCSF containing the 5-HT precursor L-tryptophan (50 μM, 10 min, $n = 7$) slightly but significantly increased the respiratory frequency to 116 ± 9%. Applying aCSF containing a 5-HT_{1A} receptor antagonist alone (either NAN190, 40 μM, $n = 10$, or p-MPPI, 40 μM, $n = 7$) for 10 min diminished the respiratory frequency to 85 ± 7% and 76 ± 8% of the control value, respectively.

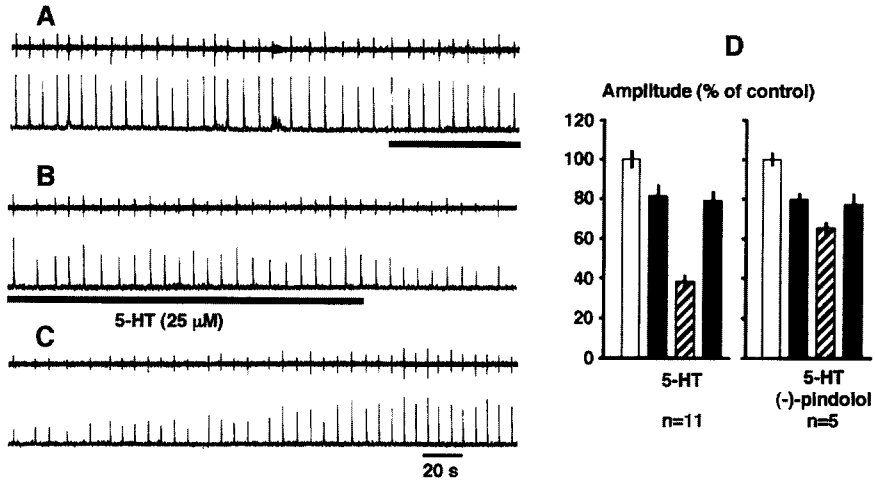

Fig. 3. Depression of the phrenic burst amplitude in response to activation of spinal 5-HT receptors. (A, B and C) Raw and integrated phrenic bursts (top and bottom, respectively) in a preparation where drugs were applied to the spinal cord alone (normal aCSF to the medulla; barrier at the level of medulla-spinal cord junction, see Fig. 1A). (A) aCSF containing NAN190 (40 μM) and SR46349B (20 μM) was applied for 10 min to block the 5-HT_{1A} and 5-HT_{2A} receptors, respectively; (B) 5-HT (25 μM, 4 min, horizontal black bar) was then applied, which depressed the phrenic burst amplitude; (C) recovery. (D) Columns and bars show the mean amplitude of the phrenic bursts and the S.E.M. calculated from identical experiments to that shown in A–C: white columns, control values under normal aCSF, grey columns, pre-treatment and recovery under aCSF containing 5-HT receptor antagonists, hatched columns, depression under aCSF containing 5-HT (25 μM). On the left, SR46349B (20 μM) and NAN190 (40 μM) were used as pre-treatment. On the right, aCSF containing SR46349B (20 μM), NAN190 (40 μM) and (−)-pindolol (40 μM) was used for pre-treatment. The phrenic burst depression was prevented by pre-treatment with aCSF containing the 5-HT_{1B} receptor antagonist (−)-pindolol.

3.2. Phrenic tonic discharge and serotonin

Applying aCSF containing 5-HT (25 μM) to the whole preparation ($n = 7$, Fig. 1B) or to the spinal cord alone (and normal aCSF to the medulla, $n = 3$) elicited a tonic discharge in the phrenic roots in 6/7 and 3/3 experiments, respectively, with a latency and a duration of about 2.5 and 5.5 min, respectively. A weaker concentration of 5-HT was less effective (in 3/7 cases when 12.5 μM of 5-HT was applied to the whole preparation). The tonic activity could also be elicited by applying to the spinal cord aCSF containing 5-HT_{2A} receptor agonists such as α-methyl-5-HT (20 μM, $n = 3/4$) and DOI (20 μM, $n = 4/5$) and could be prevented by pre-treating the preparation with the 5-HT_{2A} receptor antagonist SR46349B (20 μM, 10 min, $n = 5/5$, Fig. 3A–C). The 5-HT_{1A} receptor agonist 8-OH-DPAT (40 μM, $n = 7$, Fig. 1C) and receptor antagonist NAN190 (40 μM, $n = 5$) failed to induce and prevent the tonic discharge, respectively.

3.3. Depression of the phrenic burst amplitude by serotonin

During the occurrence of the 5-HT-induced tonic discharge, the amplitude of the phrenic bursts superimposed on the tonic discharge appeared to be depressed (Fig. 1B). This depression became more obvious when the spinal cord was pre-treated during 10 min with aCSF containing NAN190 (40 μM) and SR46349B (20 μM) to block the spinal 5-HT_{1A} and 5-HT_{2A} receptors, respectively ($n = 11$). The pre-treatment with both 5-HT_{1A} and 5-HT_{2A} receptor antagonists decreased the phrenic burst amplitude to 82 ± 6% of the control value and thereafter application of 5-HT (25 μM) reversibly depressed the amplitude of the phrenic bursts to 38 ± 3% of the control value (Fig. 3A–C). Similar depressions were also induced by the 5-HT_{1B} receptor agonists RU24969 ($n = 3$) and CGS-12066B ($n = 3$). In 5 further experiments, the 5-HT_{1B} receptor antagonist (−)-pindolol (40 μM) was added to the aCSF used for the pre-treatment (NAN190, 40 μM, SR46349B, 20 μM). As before, the pre-treatment depressed the phrenic burst amplitude to 80 ± 3% of the control value but the large 5-HT-induced depression of the phrenic burst amplitude was prevented; the phrenic burst amplitude was only depressed to 65 ± 3% of the control amplitude (Fig. 3D). If the 5-HT_3 receptor antagonist granisetron was used instead of (−)-pindolol, the 5-HT-induced depression of the phrenic bursts persisted ($n = 3$).

4. Discussion

The isolated central respiratory network of the neonatal mouse continues to elaborate rhythmic phrenic bursts in vitro with similar frequency, duration and coefficient of cycle duration variability (Di Pasquale et al., 1994) to those previously recorded in the neonatal rat. As summarized in Table 1, adding 5-HT and related agents to the aCSF superperfusing the neonatal mouse medulla-spinal cord preparation revealed that 5-HT can (1) increase the respiratory frequency, (2) induce a tonic discharge in the phrenic nerve, and (3) depress the phrenic bursts. Similar

Table 1
Summary of the subtype and location of the 5-HT receptors

	5-HT receptors involved	
	Subtype	Location
Increase in respiratory frequency	5-HT_{1A}	Medulla
Triggering of tonic spinal activity	5-HT_{2A}	Spinal cord
Decrease in amplitude the phrenic bursts	5-HT_{1B}	Spinal cord

effects were previously reported in neonatal rats (Morin et al., 1990, 1991a,b; Lindsay and Feldman, 1993; Monteau et al., 1994; Di Pasquale et al., 1992, 1994).

The pharmacological agents used in the present study were among the most well-known 5-HT tools for use with 5-HT_{1A} (8-OH-DPAT, NAN190, p-MPPI), 5-HT_{2A} (DOI, α-methyl-5-HT, SR46349B) and 5-HT_{1B} (RU24969, CGS-12066B, (−)-pindolol) receptors (Osborne, 1982; Hoyer, 1991; Zifa and Fillion, 1992; Rinaldi-Carmona et al., 1992; Hamon and Gozlan, 1993; Monteau et al., 1994; Peroutka, 1994).

In the neonatal mouse, the 5-HT-induced increase in the respiratory frequency originated from the activation of medullary receptors (double-bath experiments) of the 5-HT_{1A} subtype (effects of 5-HT_{1A} receptor agonist and antagonists). In the neonatal rat, it was concluded that endogenous 5-HT modulated the resting respiratory frequency via medullary 5-HT_{1A} receptors (Morin et al., 1990, 1991b; Monteau et al., 1994; Di Pasquale et al., 1992, 1994) since the respiratory frequency (i) increased in response to the application of 5-HT and 5-HT_{1A} receptor agonist, (ii) increased in response to an elevation of the 5-HT endogenous levels induced by activating the 5-HT biosynthesis with L-tryptophan, and (iii) decreased in response to blocking the effects of endogenous 5-HT with 5-HT_{1A} receptor antagonists. Similar results were obtained in the present study on the neonatal mouse. It can therefore be concluded that endogenous 5-HT modulates the respiratory frequency in both species.

In the neonatal mouse, the tonic firing induced in phrenic roots by 5-HT is mediated by the activation of spinal 5-HT receptors (double-bath experiments) of the 5-HT_{2A} subtype (effects of 5-HT_{2A} receptor agonists and antagonist). Similar results have been obtained on the neonatal rat, in which intracellular recordings have demonstrated that the 5-HT_{2A} receptors are located at the postsynaptic level, on the phrenic motoneuron membrane (Morin et al., 1992). In rat phrenic motoneurons, activation of these 5-HT_{2A} receptors increased the membrane input resistance, depolarised the resting potential by 20 mV and induced a tonic firing. Besides this facilitatory effect, Lindsay and Feldman (1993) reported that 5-HT decreased the phrenic inspiratory bursts by depressing the transmission of the central respiratory drive to the phrenic motoneurons. These authors hypothesized that this effect may have involved spinal non-5-HT_{2A} receptors located at the presynaptic level. Here we confirm that in the neonatal mouse, 5-HT also depresses the amplitude of the inspiratory phrenic bursts via spinal 5-HT receptors (double-bath experiments). These receptors may belong to the 5-HT_{1B} subtype, since the depression was induced by the 5-HT_{1B} receptor agonists RU24969 and CGS-12066B and blocked by the 5-HT_{1B} receptor antagonist (−)-pindolol. Due to the complexity of the 5-HT_{1B} receptor classification (Peroutka, 1994; Glennon and Dukat, 1991) no definite conclusion is possible, however, on this point and we can only rule out the possibility that 5-HT_{1A}, 5-HT_{2A} and 5-HT_3 receptors might be involved since the depression persisted when these 3 subtypes of receptors were blocked with potent selective receptor antagonists. In both rat and mouse cervical cord, 5-HT might therefore exert a subtle control on phrenic motoneuron activity by facilitating their firing via postsynaptic 5-HT_{2A} receptors and by gating the transmission of the central respiratory command, possibly via presynaptic 5-HT_{1B} receptors. This command might operate in situations where phrenic motoneurons subserve non-respiratory functions such as thermoregulation, vomiting, defecation, yawning, etc. (Monteau and Hilaire, 1991; Grelot et al., 1992).

In conclusion, the present study demonstrates that 5-HT modulates the in vitro activity of the neonatal respiratory network of mice as well as rats. In both species, 5-HT exerts a facilitatory modulation on the medullary rhythm generator via medullary 5-HT_{1A} receptors, and controls the amplitude of the inspiratory phrenic bursts via cervical 5-HT_{2A} and non-5-HT_{2A} receptors.

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