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Consequences of *In Utero* Caffeine Exposure on Respiratory Output in Normoxic and Hypoxic Conditions and Related Changes of Fos Expression: A Study on Brainstem–Spinal Cord Preparations Isolated From Newborn Rats

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ABSTRACT

Several aspects of the central regulation of respiratory control have been investigated on brainstem–spinal cord preparations isolated from newborn rats whose dam was given 0.02% caffeine in water as drinking fluid during the whole period of pregnancy. Analysis of the central respiratory drive estimated by the recording of C4 ventral root activity was correlated to Fos pontomedullary expression. Under normoxic conditions, preparations obtained from the caffeine-treated group of animals displayed a higher respiratory frequency than observed in the control group (9.2 ± 0.5 *versus* 7.2 ± 0.6 burst/min). A parallel Fos detection tends to indicate that the changes of the respiratory rhythm may be due to a decrease in neuronal activity of medullary structures such as the ventrolateral subdivision of the solitary tract, the *area postrema*, and the *nucleus raphe obscurus*. Under hypoxic conditions, the preparations displayed a typical hypoxic respiratory depression associated with changes in the medullary Fos expression pattern. In addition, the hypoxic respiratory depression is clearly emphasized after *in utero* exposure to caffeine and coincides with an increased Fos expression in the *area postrema* and *nucleus raphe obscurus*, two structures in which it is not increased in the absence of caffeine. Taken together, these results support the idea that *in utero* caffeine exposure could affect central respiratory control. **(*Pediatr Res* 53: 266–273, 2003)**

Abbreviations

∫burst, amplitude of the integrated C4 burst activity
AP, *area postrema* cNTS, commissural subnuclei of the nucleus of the solitary tract
vlNTS, ventrolateral subnuclei of the nucleus of the solitary tract
HRD, hypoxic respiratory depression
PBS, phosphate buffer saline
VLM, ventrolateral reticular nucleus of the medulla
Rf, respiratory frequency
ROb, *nucleus raphe obscurus* FLI, Fos-like immunohistochemistry
RTN, retrotrapezoid nucleus

Caffeine, which belongs to the methylxanthine family, is commonly ingested in various beverages such as coffee, tea, or cola drink (1). It interacts with adenosine neuronal transmitter system as an A_1 and A_{2A} receptor antagonist (2) and has been shown to alter the adenosinergic transmission upon chronic administration (3–5).

In rats, a positive correlation has been established between exposure to caffeine during gestation and a reduction of the fetal cerebral weight (6). Such an observation could lead to propose that caffeine modifies maturational neuronal processes. Moreover, various studies reported that a sustained maternal caffeine intake induces harmful physiologic effects on human newborns (for review, see 7). Among these effects, respiratory perturbations have been described (8–10). Nevertheless, the existence of a relation between exposure to caffeine during pregnancy and the occurrence of respiratory perturbations in

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newborns is not clearly demonstrated. Interestingly, a human newborn whose mother drank 24 cups of coffee/d during pregnancy was intoxicated by caffeine: the newborn developed apnea episodes that were attributed to the methylxanthine withdrawal (8). Abnormal behavior encountered in newborn infants has also been attributed to a large amount of maternal caffeine ingestion (10). Among the perturbations reported by these authors, periodic breathing and tachypnea are often described. Moreover, the frequency of respiratory disorders has been positively correlated with the consumption of tobacco and caffeine (9). In this latter study, however, the consequences of caffeine exposure were not easily distinguishable from those of tobacco because of the established link between tobacco and caffeine consumption. Last, the amount of apnea and periodic breathing in newborns with detectable umbilical cord blood caffeine was not different from that in similar newborns without caffeine (11). The apparent controversy between the whole of these conclusions reflects the difficulty on one hand to isolate a unique variable in research conducted in humans and on the other hand to the heterogeneity of the doses of caffeine ingested by women during pregnancy.

In the present report, our aim was to evaluate the influence of *in utero* caffeine exposure on the respiratory control in newborn rat. The use of the animal not only permits us to know precisely the dose of caffeine ingested but also ensures that the effects arise from caffeine exposure *per se* and not from anything else. To study the impact of *in utero* caffeine exposure on the respiratory control, we used a brainstem–spinal cord preparation. This model, which contains ponto-medullary areas responsible for the elaboration of the respiratory output (12–14), is particularly relevant for investigating central regulations of the respiratory control and therefore appropriate for our study. Here, we try to elucidate whether maternal caffeine ingestion during pregnancy *1*) modifies the respiratory output and *2*) induces changes in neuronal activity of areas that participate to the autonomic regulation of breathing under normoxic and hypoxic conditions. Hypoxic conditions are of interest because they *1*) are a consequence of the recurrent episodes of apnea observed in newborns and *2*) constitute a situation that requires an adaptation of the respiratory output. For these reasons, they represent a strong approach for evaluating the functionality of the neuronal mechanisms that regulate the respiratory system. Fos expression monitoring was carried out to identify the areas that presented changes of neuronal activity with regard to *in utero* caffeine exposure. Because Fos expression is activity-dependent, the detection of the Fos protein is classically used for *in vivo* studies to serve as a marker of central pathways involved in specific physiologic response as those of the respiratory response to hypoxia (15–17). Recently, we used this tool for revealing the ponto-medullary areas that could participate in the elaboration of the respiratory depression that occurs during hypoxia on brainstem–spinal cord preparations (18).

METHODS

The regional animal ethics committee approved the experimental protocol, which followed the European Communities Council Directive (86/609/EEC). Pregnant Sprague Dawley rats were divided into control ($n = 8$) and caffeine ($n = 8$) groups receiving 0.02% caffeine in water and water, respectively, as drinking fluid. The drinking fluid was exchanged every 2 d to fresh solution, and the daily intake was measured. For the caffeine group, caffeine was removed from the drinking fluid immediately after parturition. The experiments were conducted on brainstem–spinal cord preparations isolated from newborn (1–3 d) rats of control ($n = 37$) and caffeine ($n = 35$) groups. The number of pups by litter and the weight at birth were systematically recorded.

Brainstem–Spinal Cord Preparation

After a deep ether anesthesia, the CNS was isolated between the levels of the fifth cranial nerves and the C8 spinal roots, as previously described (19). The preparation was placed with the ventral surface upward in a recording chamber (volume 4 mL) continuously superfused at 26°C at a rate of 7.5 mL/min with control mock (in mM: 118.0 NaCl, 3.0 KCl, 25.0 $NaHCO_3$, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 1.0 NaH_2PO_4, 30.0 and D-glucose) adjusted to pH 7.4 by gassing with 95% O_2/5% CO_2 (control CSF).

Experimental Protocols and Data Analysis

Hypoxic tests were performed by replacing the control CSF with a CSF adjusted to pH 7.4 by gassing with a 95% N_2, 5% CO_2 gas mixture (hypoxic CSF). Two experimental protocols were conducted: one for the analysis of the respiratory output (protocol 1) and the second for the analysis of the Fos expression (protocol 2).

Protocol 1: respiratory parameters measurement. After the surgical procedure, the preparations were superfused for 30 min with control CSF. The superfusate was then replaced for 30 min by the hypoxic CSF followed by 30 min of recovery with control CSF. This procedure was conducted on brainstem–spinal cord preparations isolated from newborn rats of control ($n = 19$) and caffeine ($n = 19$) groups.

The electrical activity of the C4 ventral root was recorded using a suction electrode, filtered (10–3000 Hz), amplified (×5000), integrated (time constant 100 ms), and digitized through a Spike 2 data analysis system, with a sampling frequency of 1010 Hz. The respiratory frequency (Rf) was defined as the frequency of the bursts recorded from the C4 ventral root as established by Suzue (19). As previously reported, Rf × ∫burst (amplitude of the integrated C4 burst activity) was used as an index of the central respiratory output (20, 21). During the recording period, Rf, ∫burst and Rf × ∫burst were averaged over successive time intervals of 5 min and expressed in percentage of control values, determined during 10 min before any test. Only one trial was performed in each preparation. The hypoxic test was repeated on several preparations to determine the mean effect on Rf, ∫burst, and Rf × ∫burst.

Protocol 2, anatomical studies, tissue preparation, and immunohistochemistry. After the surgical procedure, brainstem–spinal cord preparations were either superfused with control CSF for 1 h or successively superfused with control CSF for 30 min and hypoxic CSF for the next 30 min. This

protocol was applied on preparations isolated from newborn rats of control ($n = 18$) and caffeine ($n = 16$) groups. As for the preparations used in protocol 1, the C4 electrical activity was recorded and the respiratory parameters were measured to ensure that preparations were alive. The brainstems were then transferred in a 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4), kept there for 24 h at 4°C, and finally stored at −18°C in a cryoprotectant solution for later use. Brainstems were coronally sectioned with a freezing microtome. Every second section of 40 μm was processed for Fos-like immunohistochemistry (FLI) using standard procedures. Sets of sections from control and caffeine group preparations were treated in parallel. After a 1-h blocking step in 0.1 M PBS containing 0.3% Triton X-100 and 0.5% BSA, the sections were incubated for 3 d at 4°C with a rabbit polyclonal antibody raised against Fos protein (Santa Cruz) diluted at 1/4000 in PBS with 0.3% Triton X-100 containing 0.25% BSA. Then, the sections were incubated at room temperature for 2 h in the presence of a biotinylated anti-rabbit goat immunoglobulin and for 1 h with an avidin-biotin-peroxidase complex (Vectastain Elite, Vector Laboratories). Peroxidase was detected with 0.02% 3,3'-diaminobenzidine tetrahydrochloride, 0.1% nickel ammonium sulfate, and 0.01% hydrogen peroxide in 0.05 M Tris buffer (pH 7.6). At the end of the immunohistological procedures, the sections were mounted on gelatin-coated slides, air-dried, dehydrated with absolute alcohol, cleared with xylene, and coverslipped with Depex. The specificity of the Fos antibody was checked by the supplier. Moreover, control sections were reacted without the primary antibody. Staining was absent in these conditions.

Sections were examined under the light microscope. The distribution of FLI neurons was plotted onto drawings performed with the aid of a drawing tube attached to the microscope (objective ×4 or ×10). FLI neurons were counted at higher magnification (objective ×20) in separate anatomical structures, at the same rostrocaudal levels in all the preparations, using standard landmarks (22). Cell counts were performed on the whole extent of the structures of interest. Mean number of neurons per section was calculated on one side of the neuraxis for symmetrical structures and on the whole area of medial structures.

Statistics

All values are given as mean ± SEM. The unpaired Student *t* test was used for the comparison of the drinking fluid intake between control and caffeine dams. It was also used to compare the number of pups by litter as well as the birth weight of newborn rats in control and caffeine groups. Significance was accepted at $p < 0.05$.

Protocol 1. The changes of Rf, ∫burst, and Rf × ∫burst were analyzed by comparing mean percentage values measured in successive bins of 5 min with control values. Significance was assessed using repeated measurements ANOVA followed by post hoc Fisher PLSD test. Data were regarded as significant at $p < 0.05$.

Protocol 2. Differences between the mean numbers of neurons per section and per structure obtained in the different situations were tested using one-way ANOVA followed by posthoc Fisher PLSD test. Data were considered significant at $p < 0.05$.

RESULTS

The drinking fluid intake did not significantly differ in the control (50.4 ± 5.9 mL/d; $n = 8$) and in the caffeine groups (62.3 ± 4.8 mL/d; $n = 8$). In the caffeine group, a caffeine consumption of 49 ± 4 $mg \cdot kg^{-1} \cdot d^{-1}$ can be estimated according to the drinking fluid intake. The *in utero* caffeine exposure caused a net increase in the birth weight of newborn rats [7.63 ± 0.09 g ($n = 35$) *versus* 7.08 ± 0.08 g ($n = 37$) for the caffeine and the control groups, respectively; Student *t* test, $p < 0.00001$], although it did not significantly influence the number of pups per litter (10.6 ± 2.1 *versus* 13.7 ± 0.6 pups, for the caffeine and control groups, respectively).

Spontaneous respiratory frequency. Under normoxic conditions, the mean Rf determined for preparations of the caffeine group is significantly higher [9.2 ± 0.5 bursts/min ($n = 35$); ANOVA, $p < 0.007$; Fig. 1*B*] than the one observed for control group preparations [7.2 ± 0.6 bursts/min ($n = 37$); Fig. 1*A*].

Effect of hypoxia on the central respiratory output of control group preparations. The reduction of the O_2 content of the CSF elicited a smoothly graded reduction of the central respiratory output (Figs. 1*A* and 2). During the first 5 min of hypoxia, Rf was 102.9 ± 4.5%, ∫burst was 99.9 ± 3.1%, and Rf × ∫burst was 104.4 ± 6.6% of control values. Then, a progressive decrease in Rf but not in ∫burst was observed. Six to 10 min after the beginning of hypoxic exposure, Rf and Rf × ∫burst, respectively, decreased to 89.4 ± 5.3% and 88.3 ± 5.0% of control (ANOVA, $n = 19$, $p < 0.05$), with no significant variation of ∫burst (98.0 ± 3.2% of control). This respiratory depression reached a plateau level 15 min after the onset of hypoxia (Rf 73.5 ± 5.2%, ∫burst 96.9 ± 3.8%, and Rf × ∫burst 72.8 ± 5.8% of control values). Rf and Rf × ∫burst returned to control values within 5–10 min after the end of the hypoxic test.

Effect of hypoxia on the central respiratory output of caffeine group preparations. For the caffeine group preparations, the hypoxia-evoked decrease in Rf was significantly more pronounced than for preparations of the control group (ANOVA, $n = 19$, $p < 0.002$, Figs. 1*B* and 2), although nothing significant could be seen regarding the ∫burst. The decrease in Rf and consequently in Rf × ∫burst was already observed during the first 5 min of the hypoxic exposure (Rf 64.8 ± 5.4% and Rf × ∫burst 66.4 ± 6.0% of control values).

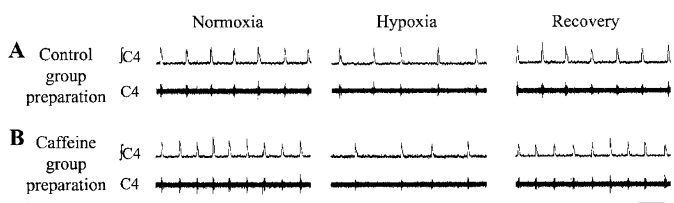

Figure 1. Respiratory C4 activity in control (*A*) and caffeine (*B*) group preparations in normoxia, 15 min after the onset of hypoxia, and in normoxia recovery. ∫C4, integrated C4 activity (upper traces); C4, C4 activity (lower traces). Scale bar = 10 s.

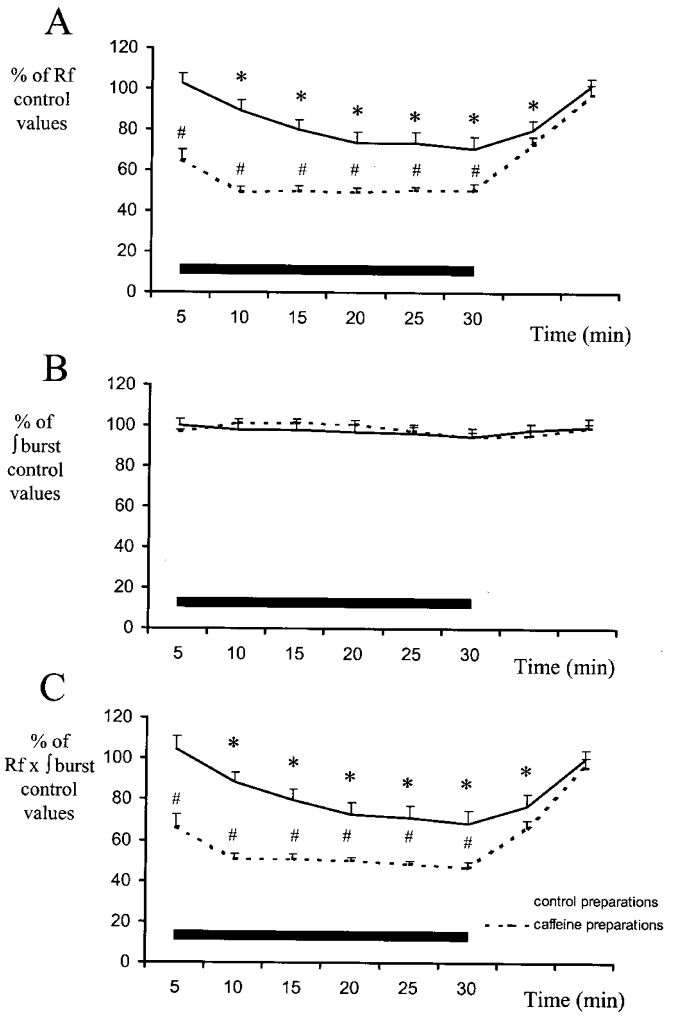

Figure 2. Effects of hypoxic exposure (black bar) on mean Rf (*A*), mean ∫burst (*B*), and mean Rf × ∫burst (*C*). Each point corresponds to a 5-min average recording period. *For the control group, a significant decrease in mean Rf (*A*) and mean Rf × ∫burst (*C*) compared with control values (ANOVA, Fisher PLSD test); #significant decrease in mean Rf (*A*) and mean Rf × ∫burst (*C*) in caffeine group compared with control group preparations (ANOVA, Fisher PLSD test).

Later, a more marked depression of the respiratory output compared with that of the control group preparations (ANOVA, $n = 19$, $p < 0.002$) was maintained to a plateau level displaying Rf, ∫burst, and Rf × ∫burst values of 49.2 ± 2.6%, 101.0 ± 2.1%, and 50.6 ± 2.7% of control, respectively. As previously, Rf and Rf × ∫burst were back to control values within 5–10 min after the end of the hypoxic test.

Fos expression in ponto-medullary areas of control group preparations. The brainstems of preparations superfused with control CSF displayed a basal Fos expression in several areas devoted to the respiratory control (Table 1, Fig. 3*A*). Superfusing the preparation with hypoxic CSF for 30 min induced significant changes of Fos expression in medullary but not pontine areas (Table 1, Fig. 3*A*). Fos expression was clearly increased in the retrotrapezoid nucleus (RTN; ANOVA, $n = 18$, $p < 0.003$), whereas it was decreased in the ventrolateral reticular nucleus of the medulla (VLM; ANOVA, $n = 18$, $p < 0.005$; Fig. 4, *A* and *B*). No changes of Fos expression were observed in other areas devoted to the respiratory control, such as the commissural and ventrolateral subnuclei of the nucleus of the solitary tract (cNTS, vlNTS), the *area postrema* (AP), the *raphe pallidus* and *raphe obscurus* (ROb), at the medullary level, and the A5 region and the subceruleus region at the pontine level.

Fos expression in ponto-medullary areas of caffeine group preparations. Significant changes of Fos expression under normoxic conditions were found in caffeine group preparations compared with control group preparations (Table 1, Fig. 3*B*). More precise, Fos expression was decreased in the vlNTS (ANOVA, $n = 16$, $p < 0.0002$), the AP (ANOVA, $n = 16$, $p < 0.008$) and the ROb (ANOVA, $n = 16$, $p < 0.008$). Furthermore, hypoxia triggered alterations in Fos expression in medullary areas not only compared with the normoxic Fos expression of this caffeine group but also compared with the hypoxic Fos expression of the control group (Table 1, Fig. 3*B*). Indeed, Fos expression level was higher in the AP (ANOVA, $n = 16$, $p < 0.004$), the ROb (ANOVA, $n = 16$, $p < 0.0005$; Fig. 4, *C* and *D*), and the RTN (ANOVA, $n = 16$, $p < 0.004$) and lower in the VLM (ANOVA, $n = 16$, $p < 0.003$) compared with normoxia. Furthermore, under hypoxic conditions, caffeine group preparations showed a decrease in Fos expression in the vlNTS (ANOVA, $n = 16$, $p < 0.001$) and an increase in the ROb (ANOVA, $n = 16$, $p < 0.02$) compared with control group preparations.

DISCUSSION

With the use of electrophysiological and immunohistological approaches, the current study provides evidence that *in utero* caffeine exposure induces changes of the central respiratory control observed on brainstem–spinal cord preparations of newborn rats.

General Observations

Caffeine exposure. It is by now established that the metabolism of caffeine differs between rats and humans: the half-life of this methylxanthine is much shorter in rats. Therefore, a correction factor relative to the metabolic body weight for the dose must be applied for comparing caffeine administration (2, 7). In this context, Tanaka *et al.* (6) demonstrated that a dose of caffeine of 70 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ ingested by pregnant rats is equivalent to a dose of ~30 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ for humans. They also concluded that a dose of caffeine comparable to what is used in this study (49 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$) is in the moderate range for a human model. Moreover, caffeine consumption by the drinking fluid is naturally divided into several takes during the day and thus facilitates the comparison with humans. This mode of administration is definitely preferable to the single high dose commonly used for experiments in rodents. It seems likely, therefore, that the parameters of drug administration that were used throughout this study are appropriate for extrapolating the results to human observations (2).

Methodological considerations. The respiratory nature of the rhythmic activity recorded from the ventral C4 root of the brainstem–spinal cord preparations isolated from neonatal rat is now clearly established (14). Moreover, the authors (23, 24)

Table 1. *Fos expression in ponto-medullary areas*

	Control group		Caffeine group	
	Normoxia (n = 9)	Hypoxia (n = 9)	Normoxia (n = 8)	Hypoxia (n = 8)
Medulla				
cNTS	31.0 ± 3.2	31.2 ± 5.7	23.3 ± 7.7	24.0 ± 5.2
v1NTS	21.1 ± 2.0	19.4 ± 2.4	7.8 ± 1.9*	9.4 ± 1.4‡
AP	17.6 ± 0.6	15.4 ± 4.8	4.9 ± 0.5*	17.7 ± 2.2†
VLM	22.9 ± 5.5	9.9 ± 1.8*	22.1 ± 1.7	8.6 ± 0.4†
RPa	7.4 ± 3.4	4.4 ± 0.9	6.6 ± 1.1	5.4 ± 0.5
ROb	24.8 ± 5.0	18.2 ± 5.2	7.7 ± 0.9*	32.3 ± 1.7†‡
RTN	7.5 ± 1.1	21.7 ± 4.1*	7.3 ± 1.1	22.4 ± 3.5†
Pons				
A5	14.1 ± 4.7	12.3 ± 2.7	9.1 ± 2.3	13.1 ± 3.4
Sub C	39.0 ± 8.2	31.2 ± 7.8	23.3 ± 6.8	26.4 ± 4.5
LC	11.3 ± 4.0	11.6 ± 2.5	8.4 ± 0.7	9.7 ± 1.0

Values are mean (±SEM) of FLI neurons/section.
* Significant change of Fos expression compared with that of the normoxic control group.
† Significant change of Fos expression compared with that of the normoxic caffeine group.
‡ Significant change of Fos expression compared with that of the hypoxic control group.

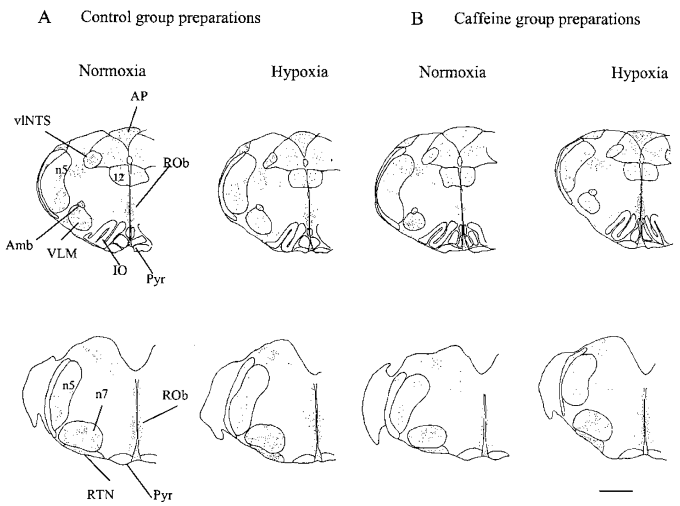

Figure 3. Distribution of Fos-positive neurons at two different rostrocaudal levels of the medulla in control group (*A*) and in caffeine group preparations (*B*). Abbreviations: Amb, *ambiguus nucleus*; IO, inferior olives; n5, trigeminal nucleus; n7, facial nucleus; Pyr, pyramidal tract; 12, hypoglossal nucleus. Scale bar = 500 μm.

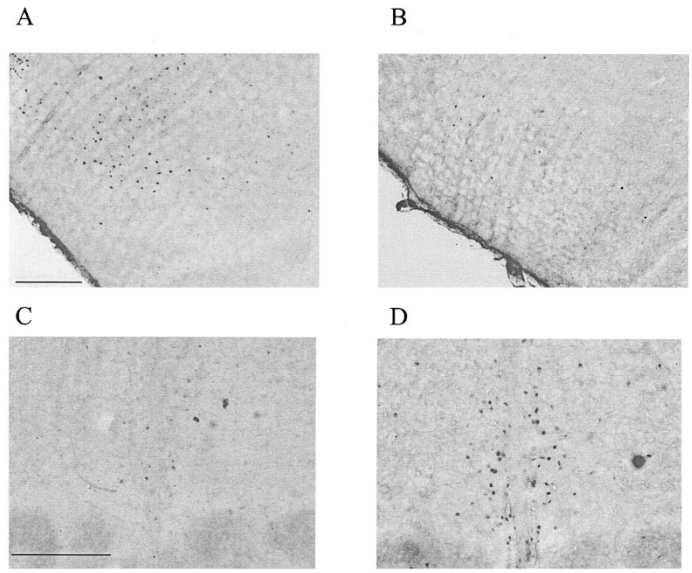

Figure 4. Fos-positive neurons in the VLM of control group preparations in normoxic (*A*) and hypoxic (*B*) conditions and in the ROb of caffeine group preparations in normoxic (*C*) and hypoxic (*D*) conditions. Scale bar = 200 μm.

who demonstrated the existence of an oxygen pressure gradient across the preparation concluded that it was reasonable to assume that respiratory neurons located superficially are functioning under aerobic conditions. The use of this preparation to investigate the respiratory control tacitly implicates that all of the peripheral and suprapontine influences are eliminated, which permits the limitation of the observations to ponto-medullary areas that are directly responsible for the elaboration of the respiratory rhythm (12, 13). Of interest is the ability of this experimental system to maintain a respiratory rhythmic activity under low O_2 level conditions for analyzing central mechanisms of the respiratory response to hypoxia. *In vivo*, hypoxia elicits a biphasic respiratory response that consists of an initial increase, followed by a reduction of the respiratory output (25, 26). The initial increase is essentially triggered by excitatory inputs from the peripheral chemoreceptors (25) with the participation of hypothalamic areas (27), whereas the hypoxic respiratory depression (HRD) seems to arise from a central origin (25) in which ponto-medullary areas seem to play a key role (28, 29). The brainstem–spinal cord preparation has been shown to respond to hypoxia with a reversible decrease in Rf and was used successfully for breaking down the ponto-medullary mechanisms underlying the HRD (18, 20, 21, 30–32). Thus, this preparation constitutes an appropriate model for investigating the influence of *in utero* exposure to caffeine on the central respiratory output of newborn rats.

The immediate early genes, such as *c-fos*, have different properties that make them suitable markers of neuronal activity (33). Detection of their expression permits the revealing of central pathways involved in specific physiologic responses, such as the respiratory response to hypoxia (15, 16, 34). In our hands, the detection of the Fos protein on the brainstem–spinal cord preparation constituted a valuable tool for determining the

whole central structures involved in the HRD (18). The same approach is used hereby with the aim of analyzing the effects of *in utero* caffeine exposure on ponto-medullary structures of newborn rats under normoxic or hypoxic conditions. In the present report, 30-min-long hypoxic stimulations were used as previously described (18). Thirty minutes of hypoxia constitutes a critical stimulus that conciliates the electrophysiological recording of HRD and a detectable Fos expression (21, 31, 32, 35, 36). Nevertheless, posthypoxic neuronal mechanisms that interact with central respiratory control have been demonstrated (37). For excluding the possibility that the changes of neuronal activity revealed by Fos detection might be related to these posthypoxic neuronal mechanisms, the immunohistological approach was conducted on a separate group of preparations (protocol 2). This group was submitted to a hypoxic period of 30 min without a recovery period.

Effect of Hypoxia on the Central Respiratory Output and Related Changes of Fos Expression

Hypoxia induced a decrease in respiratory output characterized by a diminished Rf without modification of ∫burst. This response is similar to those described in previous studies (18, 21, 31, 32) except that of Okada *et al.* (20) that reported a decrease in ∫burst. The origin of this discrepancy could rest in the pH of the CSF: the fluid used by Okada *et al.* is buffered to 7.8 instead of 7.4 in other reports, and the influence of the pH on the ∫burst has been demonstrated (38). Basically, our data show that superficial and deep ponto-medullary areas express the Fos protein under normal oxygenation conditions in the *in vitro* brainstem–spinal cord preparation. It seems reasonable, therefore, to use the Fos expression to determine areas that present any modifications of activity in response to specific stimuli. We have recently reported that hypoxic exposure of the brainstem–spinal cord preparation resulted in an increased expression of Fos in the RTN together with a decreased expression in the ventrolateral reticular nucleus VLM (18). These differences in Fos expression observed under normoxic and hypoxic conditions could, directly or not, reflect the activation of O_2-sensing neurons that have been found in the medulla (39, 40). Obviously, the absence of dramatic and aspecific changes in the expression of Fos tends to drain out the involvement of a metabolic depletion being responsible for a diminished neuronal activity. A high level of Fos expression in the RTN brings new elements in favor of a key role played by this area in the HRD. RTN neurons have been primarily reported to be involved in the central pathway activated by hypoxia (17, 29, 41, 42), and recently, they have been proposed to trigger or relay a central depressive influence of hypoxia on the respiratory network (21). Because the RTN is the only structure that presented a hypoxia-evoked elevation of Fos expression, one may suppose that either it contains O_2-sensing neurons or it is submitted to the removal of inhibitory influences exerted by other areas. In this latter hypothesis, VLM could be a suitable candidate area because it showed a reduced Fos expression under hypoxia *versus* normoxia. However, no projections arising from the VLM have been shown to reach the RTN in the rat (43), and, thus, the presence of O_2-sensing neurons in the RTN remains the most likely hypothesis. One may not exclude that O_2-sensing neurons located elsewhere could fail to express Fos but exert an excitatory influence on RTN neurons. Moreover, as the VLM includes a part of a neuronal group participating in the respiratory rhythm generation, namely the ventral respiratory group (44), the observed decrease in Fos expression under hypoxic conditions might simply be related to the decline of the respiratory output. Despite this, the identified projections from the RTN to the ventral respiratory group (43) suggest that RTN neurons may exert their inhibitory influence on the respiratory output directly on the ventral respiratory group neurons. In our preparation, the Fos protein level in pontine structures such as A5 and subceruleus regions did not vary through hypoxia, although a rise of it has been shown *in vivo* (34, 45). This suggests that the elevated Fos expression seen *in vivo* either depends on peripheral and/or suprapontine areas not present in the brainstem–spinal cord preparation or is linked with a system other than respiratory control, such as the cardiovascular system, in that they interfere (46).

Effect of the *In Utero* Caffeine Exposure on Central Respiratory Output and Fos Expression in Normoxic and Hypoxic Conditions

A maternal caffeine ingestion during gestation induced changes of the central respiratory output as well as the Fos expression observed under normoxic and hypoxic conditions.

Normoxic conditions. Under normoxia, the Rf of the brainstem–spinal cord preparations recorded from the caffeine group was higher than the one obtained with the control group. This result is in agreement with a recent report (47) and tends to indicate that an exposure to caffeine during the gestation could influence the respiratory control of newborns as hypothesized. This increase in Rf could be related to the tachypnea observed in human newborns whose mother had an important consumption of coffee while pregnant (10). Nevertheless, we did not observe any respiratory pause (apnea) as described for human newborns exposed to caffeine *in utero* (8, 10). Such perturbation could depend on the influence of caffeine on the peripheral or suprapontine components of the respiratory control that participate to the adaptation of the central respiratory output to various situations (48, 49). However, it cannot be excluded that a 30-min recording is not long enough to detect apnea. Simultaneously, the exposure to caffeine led to a smaller Fos expression at the medullary level in the AP, the vlNTS, and the Rob, which suggests a substantial decrease of neuronal activity in these areas. Together with an elevated Rf, this decrease in neuronal activity could lead to the proposal that the regions mentioned above would exert a moderator influence on the respiratory output that could be somehow removed through *in utero* caffeine exposure. In agreement with this assumption, the chemical stimulation of the AP has been demonstrated to decrease the respiratory output *in vivo* (50). Similarly, an inhibitory effect of the ROb on respiratory control has been reported *in vivo* (51). The vlNTS is a group of neurons that corresponds to the DRG initially described in the cat (52); its existence in the rat is still the subject of controversy (44).

Although there is evidence that this group of neurons does not exert any influence on the respiratory control (53), it may play a moderator role under our experimental conditions.

Hypoxic conditions. *In utero* caffeine exposure leads to a net emphasis of the HRD as well as to clear differences in Fos expression. This influence of maternal caffeine intake on the central respiratory control of the newborn was already suggested by studies in humans (8–10). Under hypoxic conditions for the preparations of the caffeine group, we observed an increase in Fos expression in the RTN and a decrease in the VLM as for control group preparations, but we also observed an elevated Fos expression in the AP and the ROb that was not present in the control group preparations. Moreover, the vlNTS and the ROb exhibited respectively a lower and a higher level of Fos expression compared with what was seen with the control group in hypoxic conditions. The higher level of Fos protein induced by hypoxia in the AP and especially in the ROb suggest that these regions are involved in the exaggeration of the HRD. Taken together with our previous results, these data tend to confirm the hypothesis according to which the AP and the ROb could exert a moderator influence on the respiratory output. Because hypoxia did not elicit any significant difference in the level of Fos expression in the vlNTS between normoxia and hypoxia for the caffeine group, it is reasonable to think that this region does not participate in the exaggeration of HRD, although a decrease in Fos expression could be detected upon hypoxia in preparations of the caffeine group compared with the hypoxic control group preparations. The difference in the respiratory output seen during hypoxia in the caffeine *versus* control group of preparations could reflect a boost of the central mechanisms responsible for the HRD. Among these mechanisms, a local accumulation of neurotransmitters such as adenosine has been proposed (26, 54). Besides, for concentrations comparable to these used throughout this study, caffeine is known essentially to interact with A_1 and A_{2a} adenosine receptors (for review, see 2). It has been reported that chronic caffeine exposure of the adult increases the number and/or the sensitivity of adenosine receptors at the central level (3–5). This latter hypothesis could be extended to newborn rats whose dam has been exposed to caffeine. However, a recent report by Aden *et al.* (55) showed that *in utero* exposure to caffeine did not modify the number of A_1 and A_{2a} adenosinergic receptors in newborn rats in various areas, including the cerebral cortex, the cerebellar cortex, the hippocampus, and the thalamus. Nevertheless, the size of these regions is relatively large and the authors do not exclude that changes may exist in definite small groups of cells. Because this study did not concern the brainstem alterations of the number of adenosine receptors, the cerebral region conserved in our preparation, changes in number of adenosine receptors is still to be considered in mediating the effects of *in utero* caffeine exposure.

In conclusion, the present report suggests that exposing a pregnant rat to caffeine leads to alterations of the central respiratory control of the newborn rats as observed on brainstem–spinal cord preparation. These alterations coincide with changes of the Fos expression pattern at the medullary level in areas devoted to the respiratory control. Of particular interest is the finding that the HRD is exaggerated after a caffeine *in utero* exposure. This observation tends to indicate that *in utero* caffeine exposure might lead to the increase in the respiratory depression encountered during hypoxic episodes consecutive to apnea in human newborns. Nevertheless, further investigations are required to extrapolated the present results to humans. For instance, an *in vivo* approach would be necessary to incorporate the influence of peripheral and suprapontine areas.

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