Prenatal Diazepam Exposure Alters Respiratory Control System and GABA_A and Adenosine Receptor Gene Expression in Newborn Rats

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ABSTRACT: In experimental animals, prenatal diazepam exposure has clearly been associated with behavioral disturbances. Its impact on newborn breathing has not been documented despite potential deleterious consequences for later brain development. We addressed this issue in neonatal rats (0–2 d) born from dams, which consumed 2 mg/kg/d diazepam *via* drinking fluid throughout gestation. *In vivo*, prenatal diazepam exposure significantly altered the normoxic-breathing pattern, lowering breathing frequency (105 vs. 125 breaths/min) and increasing tidal volume (16.2 vs. 12.7 mL/kg), and the ventilatory response to hypoxia, inducing an immediate and marked decrease in tidal volume (–30%) absent in controls. *In vitro*, prenatal diazepam exposure significantly increased the respiratory-like frequency produced by pontomedullary and medullary preparations (+38% and +19%, respectively) and altered the respiratory-like response to application of nonoxygenated superfusate. Both *in vivo* and *in vitro*, the recovery from oxygen deprivation challenges was delayed by prenatal diazepam exposure. Finally, real-time PCR showed that prenatal diazepam exposure affected mRNA levels of $\alpha 1$ and $\alpha 2$ GABA_A receptor subunits and of A₁ and A_{2A} adenosine receptors in the brainstem. These mRNA changes, which are region-specific, suggest that prenatal diazepam exposure interferes with developmental events whose impact on the respiratory system maturation deserves further studies. (*Pediatr Res* 64: 44–49, 2008)

As a benzodiazepine, diazepam enhances GABA action at GABA_A receptors (GABA_AR) by increasing the frequency of chloride channel opening (1). It may be prescribed to pregnant women for the management of anxiety, of seizures and as a muscle relaxant. Diazepam is easily transported across the placenta to the fetus, where it substantially accumulates in the brain. Epidemiologic studies on the risk of congenital malformations among children exposed to diazepam *in utero* have not been conclusive (2). Nevertheless, some reports raised the concern of delayed impairment of the child's neurobehavioral processes (3). This issue, together with that of the mechanisms involved, has mostly been addressed in rodents, to overcome confounding environmental and social variables encountered in clinical studies on substance abuse.

Most investigations supported the idea that prenatal diazepam exposure may have a detrimental impact on behavioral scores in the adulthood. Although respiratory disturbances may occur after birth in babies born to mothers treated with diazepam (2,4), no study did speculate about developmental alterations despite the importance of GABAergic system in the regulation of neonatal and adult breathing (5–7). Hence, we studied the consequences of gestational exposure to diazepam on the breathing pattern and the ventilatory responses to hypoxia in unrestrained 0–2-d-old rats (P0–P2) and on the inspiratory drive produced by brainstem-spinal cord preparations in a normal oxygenated environment and following oxygen depletion. In addition, quantitative real-time polymerase chain reaction was conducted to check whether the functional consequences of diazepam prenatal exposure might reflect changes in the expression of genes encoding for GABA_AR. We monitored the transcription levels of its $\alpha 1$ and $\alpha 2$ subunits because they are constituent of the benzodiazepine binding site, which are the most abundant in the CNS (8) and they exhibit development-dependent expression throughout areas of the brain (9). We also monitored the transcription levels of A₁ and A_{2A} adenosine receptors (A₁R and A_{2A}R) because they are downregulated by chronic administration of diazepam in the adult forebrain (10) and they are crucial for breathing control processes in newborns (11,12), partly *via* control of endogenous GABA release (13).¹

MATERIALS AND METHODS

The experiments were carried out on 0–2-d-old (P0–P2) Sprague-Dawley rats born in the local husbandry, in conformity with the European Council Directive (86/609/EEC) and approved by the local Scientific Council.

Diazepam (Roche) was continuously delivered to the dams *via* drinking fluid (0.001% in tap water) during the whole gestation and after birth. This mimics exposure conditions common in humans and avoids withdrawal reactions in newborns. Diazepam did not influence dam's fluid intake (60 ± 6 vs. 57 ± 7 mL/d in controls) and was absorbed at a daily dose of 1.87 ± 0.04 mg/kg, within the range of previous studies (14,15).

In vivo experiments. Respiratory recordings were performed as previously described in newborns (16) by whole-body plethysmography according to the barometric method.

Abbreviations: aCSF, artificial cerebrospinal fluid; A₁R, adenosine A₁ receptor; A_{2A}R, adenosine A_{2A} receptor; DZP, diazepam; GABA_AR, GABA_A receptor; Int C4, integrated amplitude of C4 burst activity; Rf-like, C4 burst frequency

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Newborns were transferred from the nest to a 60 mL recording chamber maintained at thermoneutral temperatures ($32.8 \pm 0.1^\circ\text{C}$) (17) and continuously flushed with humidified air or calibrated gas mixture. The chamber was connected to a differential pressure transducer (Validyne DP 45; sensitivity $\pm 5 \text{ cm H}_2\text{O}$) to record the pressure changes induced by the respiratory flow. Signals were digitized and stored (Spike 2, CED, Cambridge, UK) for off-line calculation of respiratory frequency, tidal volume, and minute ventilation. Newborns were allowed to adapt to the chamber for 10 min before measurements, which were performed every 5 min during 30 s runs.

To assess whether time spent in the chamber may affect respiratory parameters, basal ventilation was monitored in separate groups of controls and newborns exposed to diazepam (DZP newborns) for 75 min. The response to hypoxia (11% O_2 in N_2 during 45 min) was assessed in separate groups, relatively to prehypoxic respiratory parameters, averaged over a 15 min period. Recovery was checked over 15 min following return to normoxia.

To calculate tidal volume (18), rectal temperature in normoxia and hypoxia was measured every 5 min *via* an implantable thermoprobe (diameter 0.6 mm) in additional sets of newborn rats, to minimize handling and discomfort in rats used for respiratory measurements.

In vitro brainstem–spinal cord preparations. The brainstem and the cervical spinal cord were dissected and isolated as a single bloc as previously described (19). The preparations were transferred to a recording chamber and superfused (rate: 4 mL/min) with artificial cerebrospinal fluid (aCSF) at $27 \pm 1^\circ\text{C}$ and pH 7.4, saturated with 95% O_2 –5% CO_2 and containing (in mM): 129 NaCl, 3.35 KCl, 1.26 CaCl_2 , 1.15 MgCl_2 , 21.0 NaHCO_3 , 0.58 NaH_2PO_4 , 30 D-glucose.

Phrenic burst discharges were recorded from C4 ventral root using suction electrodes. The signal was amplified, filtered (0.1–3 kHz), integrated (time constant 50 ms), and digitized through Spike 2 data acquisition system. Data were stored for off-line calculation of the frequency of the integrated C4 burst (Rf-like) and its amplitude (Int C4). The durations of phrenic bursts and of silent periods were calculated as indexes of inspiratory (T_I) and expiratory times (T_E), respectively.

Tissue hypoxia was induced by replacing normal aCSF by nonoxygenated aCSF, bubbled with 95% N_2 , 5% CO_2 , for 30 min. This delay allowed full development of the response while minimizing risks of anoxic tissue damage (20). Thereafter, preparations were allowed to recover under normal oxygenated aCSF for 20 min.

In vivo and in vitro data analysis. For data presentation, group averages have been calculated together with standard errors of the means. Changes as a function of time were analyzed by one-way ANOVA for repeated measures, completed by Fisher *post hoc* least square difference (PLSD) test. Differences between groups were assessed using one-way ANOVA. Differences were considered significant at $p \leq 0.05$.

Gene expression studies. Receptor gene expression levels were quantified at P0 using SYBR green based quantitative real-time RT-PCR. mRNAs were quantified in the medulla and the pons, both crucial for breathing control, and in the cortex, selected for comparison with previous behavioral studies.

RNA isolation and cDNA synthesis. Tissue samples from three neonates from separate litters were pooled to correct for inter-individual variability in gene expression levels. After killing, the brain was quickly removed from the skull. The cortex of the right cerebral hemisphere, the pons and the medulla were collected in separate tubes, frozen in liquid nitrogen, and stored at -80°C . Total RNA was extracted with Trizol Reagent (Invitrogen Life Technologies) followed by DNase digestion (RQ1 RNase-Free DNase, Promega). The integrity, quantity, and purity of the RNA yields were checked by electrophoresis and spectrophotometry. First strand cDNA was synthesized at 42°C for 50 min by Superscript TM II Reverse Transcriptase (200 units; Invitrogen Life Technologies) from 1 μg of total RNA in 20 μL reaction buffer containing dNTP (500 μM ; PCR Nucleotide Mix^{PLUS}, Roche), 1.25 μM random decamers (Roche). The RNA template was degraded by *E. Coli* RNase H (2 units; Invitrogen Life Technologies).

Real time PCR. PCR products were synthesized using a Light Cycler Instrument (Roche) according to the manufacturer's instructions. Reactions

were performed in duplicate on 5 μL cDNA added to 15 μL reaction mixture, containing 0.5 μM forward and reverse primers (MWG Biotech), and 4 μL FastStart DNA master Plus mix SYBR Green I. Following denaturation (95°C , 10 min), amplification was performed for 40 cycles of 95°C for 10 s, 60 – 65°C for 10 s (annealing step at primer melting temperature, see Table 1) and 72°C for 10 s. The purity and specificity of the resulting PCR products were assessed by melting curve analysis and electrophoresis. Negative control reactions were performed in the absence of cDNA.

PCR primers were replicated from published information (18S rRNA, *ADORA1*) (21) or designed from sequence databases (NCBI, USA) using the LC Probe Design Software (Roche) (*ADORA2A*, *GABRA1*, and *GABRA2*) (Table 1).

Gene expression was normalized to 18S ribosomal RNA (18SrRNA) whose expression has been shown to be stable (21,22). Changes in gene expression levels were quantified according to Pfaffl (23), basing on the cycle threshold number (CT) and the PCR efficiency (E) of target and reference genes. The magnitude of changes is given by the ratio:

$$R = E_{\text{target}}^{\Delta\text{CT}(\text{control}-\text{treatment})} / E_{\text{ref}}^{\Delta\text{CT}(\text{control}-\text{treatment})}$$

The PCR efficiency is calculated for each set of primers from standard curves generated by serial dilutions of a sample, tested in triplicate. Intraassay variability was determined from standard curves. Interassay variability was determined from three different experiments. Variability was calculated as CT variations from CT mean value and expressed in percentage ($\text{SD} \times 100/\text{mean}$).

RESULTS

As previously shown at similar dose regimens (14,15), maternal DZP consumption had no deleterious effect on pup viability or physical development and only a minor proportion (3.4%) of the newborns used in the present experiments could be regarded as growth retarded (body weight below mean control value minus 2 standard deviations).

Effect of prenatal diazepam exposure on normoxic and hypoxic ventilation. Under normoxia, newborn ventilation stabilized within the first 15 min of recordings, without any significant change over the remaining period. Newborns exposed to diazepam (DZP newborns) exhibited a lower breathing frequency (f) than controls, because of the lengthening of both inspiration and expiration (Table 2). In contrast, tidal volume (V_T) was greater than in controls. Nevertheless, minute ventilation (V_E) was similar in both groups.

In controls, V_E displayed a biphasic response to hypoxia, representative of changes in f , which first raised to a maximum (max) within 6 ± 1 min and then dropped to a minimum (min) below normoxic values 32 ± 2 min after the onset of hypoxia (Fig. 1). At this time, rectal temperature was lower than in normoxia but the difference was not significant (34.7 ± 0.4 vs. $36.0 \pm 0.9^\circ\text{C}$). V_T did not significantly vary during hypoxia and respiratory parameters recovered basal values within 15 min after the return to normoxia.

In DZP newborns under hypoxia, f raised to max within the same delay as in controls and dropped to min at 37 ± 2 min. V_T underwent an immediate and continuous decrease (Fig. 1).

Table 1. Characteristics of the specific primers used for real-time PCR

Gene	Receptor	Forward (5'→3')	Reverse (3'→5')	bp	Annealing temp ($^\circ\text{C}$)	E
<i>GABRA1</i>	GABA _A $\alpha 1$ subunit	TTT GGA GTG ACG ACC G	CT AAT CAG AGC CGA GAA	139	60	1.91
<i>GABRA2</i>	GABA _A $\alpha 2$ subunit	TGG TGC TGG CTA ACA T	GTC CTG GTC TAA GAC GAT	108	60	1.87
<i>ADORA1</i>	Adenosine A ₁	GGA TCG ATA CCT CCG AGT CA	GAG AAT CCA GCA GCC AGC TA	116	65	2.01
<i>ADORA2a</i>	Adenosine A _{2A}	AGT CAG AAA GAC GGG AAC	CAG TAA CAC GAA CGC AA	120	60	2.03
18S rRNA		CTT AGA GGG ACA AGT GGC G	GGA CAT CTA AGG GCA TCA CA	67	60	1.93

E, efficiency; bp, base pair.

Table 2. Influence of diazepam exposure on basal respiratory parameters in intact newborn rats

	N	f (cycles/min)	T _I (s)	T _E (s)	V _T (mL/kg)	V _E (L/min/kg)
Control	12	125 ± 5	0.13 ± 0.01	0.36 ± 0.02	12.7 ± 1.1	1.63 ± 0.19
DZP	9	105 ± 3*	0.16 ± 0.01*	0.42 ± 0.01*	16.2 ± 1.2*	1.67 ± 0.09

Values are means ± SE.

f, breathing frequency; T_I, inspiratory time; T_E, expiratory time; V_T, tidal volume; V_E, minute ventilation.

* Significant difference from control group.

Figure 1. Respiratory response to hypoxia in controls (□, n = 9) and diazepam-exposed newborns (■, n = 10). Data are expressed as % of prehypoxic value (mean ± SE) at the peak of frequency (max), at the nadir of secondary decline (min) and after 15 min of recovery under normoxia (rec). * and § indicate significant differences from baseline and control group, respectively.

This was not associated to changes in rectal temperature ($35.5 \pm 0.4^\circ\text{C}$ at min vs. $35.1 \pm 0.4^\circ\text{C}$ in normoxia). As a consequence of opposite *f* and *V_T* variations, *V_E* did not significantly increase in the early phase of hypoxia. Furthermore, respiratory parameters did not return to baseline 15 min after recovery in normoxia. This did not impair newborn's survival once back to the nest.

Effect of prenatal diazepam exposure on respiratory-like C4 activity in isolated brainstem-spinal cord preparations. We analyzed respiratory-like C4 activity (Rf-like) produced by isolated preparations before and after removing the pons which exerts an inhibitory drive to the medullary network associated to respiratory rhythmogenesis (19) (Fig. 2).

Pontomedullary preparations. Preparations from DZP newborns differed from controls by a higher Rf-like, due to both shorter inspiratory bursts (*T_I*) and silent periods (*T_E*), and higher incidence of double inspiratory bursts (Table 3).

Medullary preparations. Following pons removal, the difference in Rf-like between DZP and control preparations was reduced and mostly due to *T_I* shortening (Table 3). Double inspiratory bursts still occurred in exposed preparations while they quite disappeared in controls.

Medullary preparations under nonoxygenated aCSF. The effect of O₂ depletion was monitored on medullary preparations to focus on circuits primarily involved in respiratory rhythmogenesis.

Whatever the prenatal situation, O₂ depletion markedly reduced Rf-like (Figs. 3 and 4), as initially described (24). In controls, the drop of Rf-like was due to significant *T_E* lengthening (>300%) and *T_I* shortening (−34%). Although Rf-like decreased, Int C4 moderately increased above basal values (Fig. 4). The net effect of O₂ depletion was a major decrease in the central respiratory output (Rf-like × Int C4) within 5–30 min of hypoxia. Upon return to normal aCSF, all parameters returned to baseline within 20 min.

Figure 2. Examples of C4 burst activity in pontomedullary- (A) and medullary-spinal cord preparations (B) isolated from control and diazepam-exposed newborns. Note the higher incidence of double-bursts in the latter. Upper and lower traces: integrated and raw C4 activities.

In DZP preparations, the drop of Rf-like in response to O₂ depletion was attenuated compared with controls (Fig. 4), mostly because a reduced increase in *T_E* (160% vs. >300%). Upon reoxygenation, these preparations recovered at the same rate as controls during the first 10 min. Thereafter, Rf-like reached a plateau below baseline, *T_E* remaining 30% above basal value. Furthermore, Int C4 decreased below baseline. Consequently, the central respiratory output did not fully recover within the observation delay.

Effect of diazepam prenatal exposure on GABA_A and adenosine receptor gene expression. All primer sets enabled specific amplification. For each gene analyzed, PCR produced a single sequence at a specific melting temperature and with the expected length (Fig. 5B). All sequences amplified with high efficiency (Table 1) and with low intraassay (≤5%) and interassay variability (≤8%).

In samples from DZP newborns, the deviation of CT from control value ($\Delta\text{CT}_{18\text{S}(\text{control-dzp})}$) was -0.4 ± 0.8 for 18S

Table 3. Effect of prenatal diazepam exposure (DZP) on respiratory-like C4 activity in brainstem–spinal cord preparations

	N	Rf-like (bursts/min)	T_I (s)	T_E (s)	Double bursts (per h)
Pontomedullary-spinal cord preparations					
Control	27	4.2 ± 0.3	0.87 ± 0.07	15.1 ± 1.4	36.5 ± 9.2
DZP	23	5.8 ± 0.4*	0.72 ± 0.03*	10.4 ± 0.7*	75.0 ± 17.0*
Medullary-spinal cord preparations					
Control	29	11.7 ± 0.6	1.10 ± 0.06	4.0 ± 0.2	0.5 ± 0.3
DZP	22	13.9 ± 0.5*	0.87 ± 0.03*	3.6 ± 0.2	23.4 ± 8.7*

Values are means ± SE. Double bursts, two bursts occurring with a delay < mean cycle duration/2.

Rf-like, burst frequency; T_I , inspiratory burst duration; T_E , silent period.

* Significant difference from control group.

Figure 3. Examples of C4 burst activity in normal aCSF (O_2 aCSF), 25–30 min after O_2 depletion (non- O_2 aCSF) and after 15–20 min recovery. Note the lesser decrease in frequency and the incomplete recovery in preparations from diazepam-exposed newborns (B), compared with controls (A).

rRNA (mean ± SD, $n = 3$). This indicated that DZP exposure had no consistent effect on the expression of 18S rRNA and that it might be used to normalize changes in target gene expression. These changes might be regarded as significant ($p < 0.05$) provided $\Delta CT_{\text{target(control-dzp)}}$ was < -2 or > 1.2 (mean $\Delta CT_{18S(\text{control-dzp})} \pm 2 \times \text{SD}$).

The expression of *GABRA1*, *GABRA2*, *ADORA1*, and *ADORA2A* was detected in both controls and DZP newborns and in all regions studied. The treatment had variable effects on gene expression, depending on the target and the region studied (Fig. 5A). The expression of *GABRA1* and *GABRA2* did not significantly change in the medulla and markedly decreased in the pons and in the cortex. The expression of

ADORA1 was enhanced in the medulla, reduced in the pons and unchanged in the cortex. The expression of *ADORA2A* was reduced in the medulla and the cortex and unchanged in the pons.

DISCUSSION

We demonstrated that prenatal exposure to diazepam at a dose clinically relevant affects the pattern of resting ventilation, the central respiratory network activity and the ability of the newborn rat to cope with alveolar and tissue hypoxia both *in vivo* and *in vitro*. In addition to marked regional changes in the expression of genes encoding for GABA_A subunits constitutive of the benzodiazepine-binding site, the expression of A_1R and $A_{2A}R$ genes was altered too. This provided novel evidence that prenatal exposure to diazepam influences the development of neural networks, possibly interfering with the development of adenosinergic control of GABA release which modulates respiration in maturing rats (13).

The dose of diazepam ingested by pregnant dams was the most commonly used to induce behavioral alterations in the adult progeny without side-effects on physical development (14,15). Although higher than those used in pregnant women, it should be regarded as relatively small as the elimination half-life of diazepam and its metabolites is much shorter in adult rats than in human adults (25).

Until now, very few animal studies on the developmental consequences of prenatal diazepam exposure were concerned with the early postnatal development (26,27) and none with respiration. Impregnation or withdrawal have been regarded as the major cause of hypoventilation and apneas in human neonates exposed to diazepam at the end of fetal life (2,4). As such symptoms were not observed in DZP newborns, impreg-

Figure 4. Effect of O_2 depletion (30 min, black bar) on mean Rf-like (A), mean ln C4 activity (B) and mean respiratory output (Rf × ln C4) (C) in medullary–spinal cord preparations from controls (□, $n = 11$) and diazepam-exposed newborns (■, $n = 12$). Each point is the average of 5-min recordings. * and § indicate significant differences from baseline and control group, respectively.

Figure 5. (A) Effect of prenatal diazepam on receptor gene expression levels. Columns represent mean deviation from control level $\pm$ error estimated from maximal interassay variability. * indicates significant change. (B) Gel electrophoresis of PCR products, showing their purity and specificity. M, 25 bp-DNA ladder. NC, negative control.

nation was unlikely responsible for the observed changes in breathing pattern. Moreover, maintaining lactating dams under treatment prevented withdrawal.

Under normoxia, diazepam exposure had contrasting effects on respiratory activity *in vivo* and *in vitro*. This difference may reside on the contribution to breathing regulation *in vivo* of peripheral afferent systems eliminated *in vitro*. *In vivo*, increased T_I , V_T , and T_E may indicate alterations of vagal reflexes crucial for neonatal breathing (28). *In vitro*, data indicated that diazepam might also affect the function of central networks involved in respiratory rhythmogenesis and/or its regulation. Conceivably, dysfunction of phase-switching mechanisms may contribute to the shortening of *in vitro* respiratory cycle phases and the increased occurrence of double inspiratory bursts which are signs of respiratory instability in the newborn (29). This might partly explain the stronger impact of diazepam on preparations with the pons, which contains more neurons engaged in respiratory cycle regulation than the medulla (30).

Alveolar hypoxia evoked in newborns a typical biphasic respiratory response. The stimulation of carotid chemoreceptors is responsible for the initial hyperventilation, whereas the secondary depression is centrally triggered (31). As respiratory frequency similarly evolved in either group, diazepam was unlikely to affect the peripheral O_2 -sensitive chemoreceptor pathway. In contrast, DZP newborns differed from controls by the earlier and greater decline of V_T . Besides developmental alterations, residual diazepam bound to $GABA_A$ R in the nucleus tractus solitarius (NTS) might potentiate the depressant effects of GABA release on hypoxia-induced increase in V_T , as reported in adult rats (32).

Lacking from peripheral chemoreceptor afferents, medullary preparations enabled reliable studies of the central mechanisms of respiratory depression triggered by tissue O_2 depletion (20,24). Diazepam exposure attenuated this depression, suggesting that the chronic manipulation of $GABA_A$ R *in utero* may influence the development and/or the sensitivity of medullary O_2 -sensitive processes active under severe hypoxia.

The present findings that the expression of genes encoding $GABA_A$ R, A_1 R and A_{2A} R was altered in a region-specific manner but not that of 18S rRNA indicate that prenatal diaz-

epam may interfere with the development of distinct neurotransmitter/neuromodulator systems (10,33–35) rather than diffusely with DNA transcription or RNA stability. Our data on $\alpha 1$ $GABA_A$ R subunit mRNA expression are in agreement with adult data showing its downregulation in the cortex following chronic DZP exposure (36). In contrast, upregulation have been reported in the whole brainstem of late fetal rats following prenatal diazepam (37). As the consequences of such exposure on $GABA_A$ R distribution were region-specific and age-dependent in young rats (38), both the developmental stages and the regions studied (whole brainstem vs. pons and medulla) may account for this discrepancy, in addition to dosage protocols (s.c. vs. oral administration) and gestational periods covered by drug exposure (last week vs. full gestation).

The present data only partly corroborate previous findings that chronic diazepam may downregulate A_1 R and A_{2A} R expression (10). Indeed, it is unclear why it had quite opposite effects in the medulla, although this underscores the probable complexity of the events leading to alterations of A_1 R and A_{2A} R mRNAs. Among hypotheses, manipulation of $GABA_A$ R during early development may alter later neural organization by interacting with the production of trophic factors such as BDNF (39) which contributes to regulate adenosine receptor expression (40,41).

Changes in receptor mRNA levels supported the hypothesis that alterations in the development of GABAergic and adenosinergic systems and/or their interactions might be responsible for respiratory disturbance in the DZP newborn. For instance, decreased A_{2A} R expression may lead to inhibition of GABA release (13), which, together with decreased $GABA_A$ R expression, may depress GABAergic transmission. This might contribute to decreased respiratory frequency and shortened C4 inspiratory bursts in DZP newborns, as suggested in GAD-deficient newborns (5). In contrast, it is unclear how opposite changes in A_1 R expression in the pons and the medulla may be reflected in the respiratory adaptation to prenatal diazepam. In fact, a major pitfall in correlating respiratory alterations with $GABA_A$ R and adenosine receptor expression resides in their involvement at multiple levels of breathing control systems, which makes the balance between distinct developmental events hard to establish. Moreover, it

would be important to determine to what extent changes in definite receptor mRNA levels may combine with changes in protein, thereby resulting in alterations of receptor availability and/or sensitivity (36).

In conclusion, considering that in rats, brain maturation at birth is delayed compared with humans (42), the present observations may have a great implication for human preterm infants. Notably, the impact of prenatal diazepam on the expression of A₁R and A_{2A}R should be emphasized because it may result in unexpected responses to the treatment with their antagonist caffeine, commonly used to prevent apneas in the premature human neonate (43). Moreover, the present investigation raises the concern that chronic exposure to diazepam during pregnancy may aggravate the detrimental effects of hypoxic injury. Finally, it seems important to further assess the impact of prenatal diazepam in developing rats, because developmental changes in the expression of GABA_AR subunits are crucial for the maturation of breathing (44).

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