ARTICLES

Perinatal Respiratory Control and Its Modulation by Adenosine and Caffeine in the Rat

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ABSTRACT

The perinatal development of respiratory rhythm generation and its modulation by adenosinergic drugs have been examined in rats from embryonic d 18 (E18) to postnatal d 3 using an *in vitro* brain stem–spinal cord preparation. Generation of rhythmic respiratory activity in the medulla oblongata and inhibition of this activity by pontine structures were evident on E18. The adenosine A_1-receptor agonist, N^6-(2-phenylisopropyl) adenosine, R (−) isomer (R-PIA) (1 μM), induced an age-dependent reduction of respiratory frequency that could be reversed by the adenosine antagonist theophylline (55 μM). The effect of R-PIA was reduced 24 h after birth compared with E21 and 2 h postnatal age. In preparations from pups that had been exposed to a low dose of caffeine (0.3 g/L in drinking water to dams), pontine inhibition of respiratory rhythm generation in the medulla was more pronounced. When the pons was removed, the respiratory frequency was higher than in the control group. Adenosine A_1-mRNA and A_1-receptor development in pons and medulla were studied, and by E18, mRNA, receptor protein, and functional coupling to G-proteins were confirmed using guanylyl-5'-O-(γ-[^{35}S]thio)-triphosphate binding. There were no major changes in receptor numbers or distribution of A_1 receptors or mRNA in rat pups subjected to caffeine exposure. We conclude that respiration is already modulated by adenosine A_1 receptors at the level of the medulla oblongata in the fetal period in an age-dependent manner. Furthermore, long-term maternal caffeine intake during gestation seems to increase the pontine inhibition of, and the activity of, respiratory rhythm-generating neuronal networks in medulla oblongata without detectable changes in expression of A_1-receptor number or A_1-receptor mRNA. (*Pediatr Res* **51: 4–12, 2002**)

Abbreviations

CPA, N^6-cyclopentyladenosine
CPG, Central Pattern Generator
DPCPX, 8-cyclopentyl-1,3-dipropylxanthine
f_R, frequency of respiratory activity (bursts/min)
T_i, inspiratory time
R-PIA, N^6-(2-phenylisopropyl) adenosine, R (−) isomer
aCSF, artificial cerebrospinal fluid
E, embryonic day
P, postnatal day
GTPγ[^{35}S], guanylyl-5'-O-(γ-[^{35}S]thio)-triphosphate
CV, coefficient of variation

Fetal respiratory movements are episodic and progressively more inhibited toward the end of pregnancy when periods without respiratory movements dominate (1). Respiratory activity must be generated continuously from birth. New tactile stimuli, such as light, cooling, removal of the umbilical circulation, arousal, air in the upper and lower airways, increased pulmonary blood flow, increased oxygen consumption, vagal input from mechanoreceptors, are all involved in the initiation and maintenance of breathing (2). This transition of respiratory control is probably related to modulatory factors affecting the CPG for respiration located in the brain stem (rostral ventrolateral medulla oblongata).

Adenosine is one of the neuromodulators involved in respiratory control and may be especially important around birth (3–5). Centrally applied adenosine analogs depress respiratory rate and depth (6–8) via adenosine A_1 receptors in the brain stem (5, 9). Circulating levels of adenosine decrease immediately after birth (3), correlating to decreased extracellular levels in the brain (10), and a decrease of adenosinergic inhibition may contribute to the establishment of postnatal breathing.

Furthermore, the effect of adenosine is antagonized by caffeine in doses resembling those daily consumed by humans

(11). High doses of caffeine intake during gestation or the early postnatal period have been shown to alter adenosine receptor development (12), affect postnatal behavior, and increase the incidence of apnea in rats (11, 13–16). Po_2 during fetal life is lower than after birth. This will increase adenosine levels (17) and also the activity of pontine centers (18), both of which have been shown to depress respiratory centers in the medulla oblongata (8, 19). The possible roles of a decreased adenosinergic and pontine inhibition in the change of respiratory control after birth are not known.

The present study was undertaken to investigate the perinatal transition of the respiratory control and its modulation by adenosine. We were particularly interested in studying the effects of adenosine on medullary (CPG) and pontine structures involved in respiratory control. To study the effects of long-term adenosine receptor modulation (blocking), the fetuses and neonates were exposed to caffeine. This is also interesting as many human fetuses and neonates are exposed to methylxanthines in doses that may cause both short- and long-term effects on adenosine receptor expression and behavior. To compare central respiratory control before and after birth an *in vitro* brain stem–spinal cord preparation from fetal (20, 21) and neonatal rats was used. To examine a structural correlate to these functional studies of adenosine in the developing respiratory system, we also studied the ontogeny of adenosine A_1 receptors and their functional coupling to G-proteins and A_1-receptor mRNA in pons and medulla.

We show in the present paper that even though the isolated respiratory CPG does not undergo major changes at birth, the ability of adenosine A_1 receptors to inhibit the respiratory rhythm decreases within 24 h after birth. Furthermore, in pups exposed to a low dose of caffeine, the pontine inhibition of brain stem respiratory rhythm is increased.

METHODS

Experimental Animals

Timed-pregnant Wistar rats and their litters were used. Dams were given caffeine (Sigma Chemical Co., St. Louis, MO, U.S.A.; 0.3 g/L; $n = 23$) in the drinking water, exchanged every third day to fresh solutions, from E 2 throughout gestation and postnatal life. Twenty-one dams received ordinary tap water. The day when a vaginal plug was found was designated E0. The regional animal ethics committee approved the experiments, which followed the European Community regulations.

Brain Stem–Spinal Cord Preparation

The brain stem and spinal cord of E18 and E21 fetal rats and 2-h, 24-h, and 72-h (P3) postnatal Wistar rats ($n = 76$) were dissected and isolated as previously described (5, 22). One fetal rat from each litter was killed immediately (<60 s) after decapitation and cesarean section had been performed on the mother. The preparation was continuously perfused at a rate of 3.0–3.5 mL/min in a 2-mL chamber with the following aCSF (mM): NaCl, 124; KCl, 5.0; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 30; equilibrated with 95% O_2 and 5% CO_2; at $27 \pm 0.5°C$, pH 7.4. Forty-four preparations were

decerebrated rostral to cranial nerve V, and recordings of respiratory activity were performed with the pons remaining during perfusion with normal aCSF. A pontomedullary transection was then performed in all preparations ($n = 76$), rostrally decerebrating the brain stem between cranial nerve root VI and the lower border of the trapezoid body.

Respiratory activity of the preparation was recorded from the C4 or C5 ventral roots (C4) using suction electrodes. In some experiments ($n = 20$) the unit activity of respiratory-related neurons in the ventrolateral medulla was also recorded extracellularly using a glass microelectrode filled with 2% pontamine sky blue in 0.5% sodium chloride (resistance, 2–6 $M\Omega$). The electrode was inserted through the ventral surface into the rostral ventrolateral medulla with a micromanipulator (MW-4, Narishige, Tokyo, Japan). Respiration-related neuronal activity was found 50–300 μm below the surface (23).

Signals were amplified and band-pass filtered (10 Hz to 5 kHz, differential AC amplifier model 1700, A-M Systems, Everett, WA, U.S.A.). The C4 activity was rectified and integrated with a time constant of 100 ms. These activities were continuously monitored, registered with a strip-chart recorder, digitized continuously or in 2- to 3-min intervals/10 min, at 1–5 kHz, and stored in a computer for off-line analysis. f_R (bursts/min) and T_i (ms) were calculated from the mean C4 burst interval and C4 burst width, respectively, during 2–5 min of recording.

Drugs

The metabolically stable adenosine A_1 receptor agonist R-PIA (RBI, Natick, MA, U.S.A.) was dissolved in 5% DMSO and then further diluted in standard solution to 1.0 μM. The adenosine receptor antagonist theophylline (Sigma Chemical Co.) was diluted in standard solution to a final concentration of 20 mg/L from a 1 mM stock solution in ethanol. The final concentrations of DMSO or ethanol in the solutions were <0.01%, and control experiments with only DMSO or ethanol added to the standard solution did not change the respiratory activity. Drug solutions were prepared freshly to ensure full potency of drug effect. All drugs were added to the aCSF, and the pH was equilibrated to 7.4 with 95% O_2 and 5% CO_2 before application to the preparation, which was started by switching perfusion from normal to drug-containing aCSF. Sufficient time to reach steady-state drug response (R-PIA, 20 min, and theophylline, 15 min), as previously determined (5, 9), was allowed before evaluation of drug effect was performed.

Receptor Autoradiography and *In Situ* Hybridization

Rat brains were examined at E14, E18, E21, exactly 2 h and 24 h after vaginal delivery, and P3, P7, and P14. From each treatment group six animals (from two litters) were used for *in situ* hybridization and receptor binding studies. From embryos and pups until P3 the whole head was frozen whereas in older animals the brain was rapidly dissected out. Heads and brain were then frozen on dry ice and stored at $-20°C$. Sagittal sections from the left hemisphere were cut using a Leitz cryostat. Sections were collected from the lateral part of the

olfactory bulb toward the midline. For *in situ* hybridization, 14-μm-thick sections were thaw-mounted on poly-L-lysine (50 μg/mL) -coated slides, and for autoradiography 10-μm-thick sections were thaw-mounted on gelatin-coated slides. Specimens from different ages were processed in the same *in situ* hybridization and receptor binding runs to allow comparison of signals and binding density.

Adenosine A_1-receptor autoradiography. Receptor density was determined using receptor autoradiography with the adenosine A_1 receptor antagonist [³H]DPCPX (0.5 nM), and nonspecific binding was determined using R-PIA (100 μM). Ten-micrometer sections were preincubated in 170 mM Tris-HCl buffer containing 1 mM EDTA, 2 U/mL adenosine deaminase, and 1 mM $MgCl_2$ at 37°C for 30 min. Sections were then washed twice for 10 min at 23°C in 170 mM Tris-HCl buffer. Incubations were performed for 2 h at 23°C in 170 mM Tris-HCl buffer containing [³H]DPCPX (120 Ci/mmol, 0.5 nM), 2 U/mL adenosine deaminase, and 100 μM GTP. The incubation with DPCPX was performed in the presence of GTP to convert all of the receptors to the low-affinity state for agonists and thereby remove all endogenous adenosine (24). Sections were then washed twice for 5 min each in ice-cold Tris-HCl, dipped three times in ice-cold distilled water, and dried at 4°C using a strong fan. Slides were exposed to ³H film (Amersham Pharmacia Biotech, Uppsala, Sweden) with ³H microscales for 4 wk.

Adenosine A_1 receptor agonist-stimulated GTPγ[³⁵S] autoradiography. G-protein coupling of adenosine A_1 receptors during development was determined using GTPγ[³⁵S] autoradiography (25) with the adenosine A_1 receptor agonist CPA (30 nM). Ten-micrometer-thick sections mounted on gelatin-coated slides were incubated in 50 mM Tris-HCl buffer (pH 7.7) containing 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl at 25°C for 10 min. Slides were then incubated in Tris-HCl buffer containing GDP (1 mM) at 25°C for 25 min. Incubations were performed for 2 h at 25°C in 50 mM Tris-HCl buffer containing GTPγ[³⁵S] (1250 Ci/mmol, purchased from PerkinElmer Life Science Products, Boston, MA, U.S.A., 0.04 nM), GDP (30 mM), and (CPA, 30 mM). The basal activity was assessed with GDP in the absence of A_1 receptor agonist, and nonspecific binding was assessed in the presence of the GTP analog Gpp(NH)p (100 μM). Sections were then washed twice for 10 min each in ice-cold Tris-HCl, dipped three times in ice-cold distilled water, and dried at 4°C using a strong fan. Slides were exposed to Hyperfilm β-max (Amersham, Pharmacia Biotech, Uppsala, Sweden) for 24 h.

In situ *hybridization.* The mRNA for the A_1 receptor was identified with an antisense oligonucleotide probe. The 48-mer A_1 adenosine receptor probe was complementary to nucleotides 985–1032 of the rat A_1 receptor (26). The probe has been tested for specificity (27). The oligodeoxyribonucleotides were radiolabeled using terminal deoxyribonucleotidyl transferase and ³⁵S-dATP to a specific activity of about 10^9 cpm/μg. Slide-mounted sections were hybridized in a cocktail containing 50% formamide, 4× saline-sodium citrate, 1× Denhardt's solution, 1% sarcosyl, 0.02 M $NaPO_4$ (pH 7.0), 10% dextran sulfate, 0.06 M dithiothreitol, 0.5 mg/mL sheared salmon sperm DNA, and 10^7 cpm/mL of probe. After hybridization for

15 h at 42°C, the sections were washed four times in 1× saline-sodium citrate at 55°C, then dipped briefly in water, 70%, 95%, and 99.5% ethanol, and air-dried. Finally, the sections were apposed to Hyperfilm β-max for 4 wk.

Image analysis. Analysis of receptor expression and binding was performed using a computerized image-analysis system (Imaging Systems, St. Catherines, Ontario, Canada). Relative OD of expression or binding was measured in autoradiographs, and amounts of receptor-bound radioactivity of the specific brain regions were determined using ³H microscale standards (Amersham Pharmacia Biotech). Specific binding was calculated by subtraction of the OD values in sections in which nonspecific binding was determined. Different regions of the brain in the prenatal rats were identified using the atlas by Altman and Bayer (28) and in postnatal rats using atlases by Sherwood and Timiras (29) and Paxinos *et al.* (30).

Statistical Analysis

Off-line analysis was performed using a personal computer and the commercially available programs Axoscope and Axotape (Axon Inc., Foster, CA, U.S.A.), Origin (Microcal Software Inc., Northampton, MA, U.S.A.), and JMP (SAS Institute Inc., Cary, NC, U.S.A.). The results are presented as mean ± SEM. After ANOVA by the F test, statistical analysis was performed by multivariate analysis of variance (MANOVA) repeated-measure design, two-tailed paired t test, or Wilcoxon signed rank test when variance was unequal. Tukey-Kramer's HSD test was used to compare multiple means. Spearman rank nonparametric correlation was performed on variables with respect to postnatal age, to evaluate a possible age-dependency of the results. Quantitative receptor autoradiography and *in situ* hybridization results were analyzed by MANOVA using the procedures in the SYSTAT program (HALLOGRAM Publishing, Aurora, CO, U.S.A.). All these measurements were performed on sections from six animals. A $p \leq 0.05$ was considered to be statistically significant.

RESULTS

Development of brain stem respiratory rhythm. An irregular rhythmic respiratory activity was established in all preparations at E18 (Fig. 1*A*). The f_R at E18 was slower than at all other ages ($p < 0.05$, Tukey-Kramer's HSD test) The regularity of respiratory activity, measured as a decrease in the CV of the interval between C4 burst discharges, increased with embryonic and postnatal age ($p < 0.0001$, age *versus* CV Spearman rank correlation; Fig. 1*B*). T_i in each C4 burst increased with embryonic age (E18, 414 ± 90 ms; E21, 641 ± 135 ms; P2h, 819 ± 81 ms; P24h, 860 ± 57 ms; P72h, 812 ± 91 ms; $p < 0.05$, ANOVA). At E21 a respiratory pattern resembling that after birth had been established with regard to pontine inhibition, control f_R, and T_i. When comparing the central respiratory rhythm generated before birth (E21) with that generated 2 and 24 h after birth, no significant differences were found.

In preparations in which recordings initially were performed with the pons remaining ($n = 44$, E18–P3), removal of the pons resulted in a significant increase in f_R (from 2.3 ± 0.3 to

A

C4

B

10 s
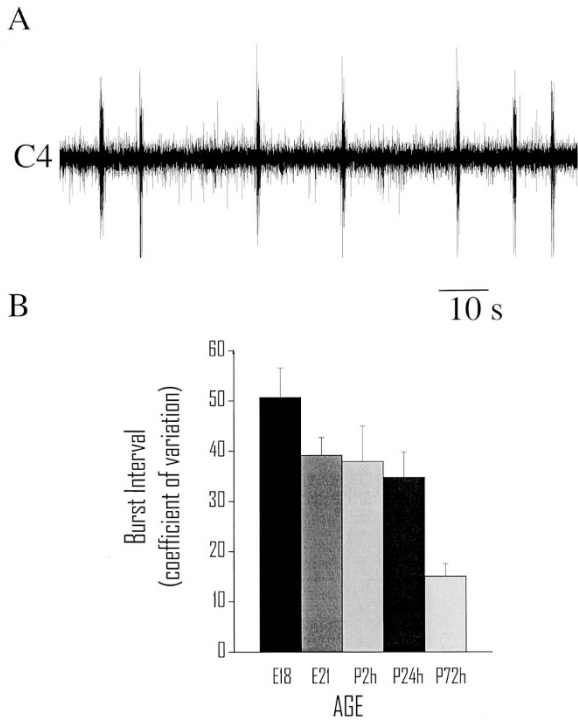
Figure 1. Development of brain stem respiratory rhythm generation. *A*, continuous but irregular C4 respiratory frequency and burst width from a fetal (E18) brain stem–spinal cord preparation without the pons. *B*, regularity of respiratory rhythm quantified as CV of C4 burst interval (mean $\pm$ SEM). Regularity of rhythmic respiratory activity increased with postnatal age (age *vs* CV, Spearman rank correlation $p < 0.0001$).

9.1 ± 0.3 bursts/min; $p < 0.0001$, paired *t* test; Fig. 2*A*). Mean f_R did not differ between age groups when the pons remained (Fig. 2*B, top*), but was significantly slower at E18 after pontomedullary transection compared with all other age groups ($p < 0.01$, Tukey-Kramer's HSD test; Fig. 2*B, middle*).

Effect of R-PIA on medullary respiratory rhythm. In the experiments in which brain stem respiratory-related neuronal activity was measured, it decreased after administration of R-PIA (1 μM) in parallel with C4 activity, as evident in Figures 3 and 4. These depressant effects on respiratory activity were reversed by theophylline (55 μM). The effect of R-PIA was age-dependent ($p < 0.0001$, MANOVA, repeated-measures design) with less f_R reduction with increasing fetal and postnatal age. The effect of R-PIA was significantly reduced 24 h after birth compared with E21 and 2h ($p < 0.01$, Tukey-Kramer's HSD test).

Caffeine exposure and development of respiratory rhythm. Caffeine-exposed pups did not differ from controls with respect to litter size or weight. The pontine inhibition of brain stem–generated C4 activity was more pronounced in the caffeine group ($p < 0.05$, MANOVA, repeated-measures design; Fig. 2*B*). With the pons removed, brain stem–spinal cord preparations from caffeine-exposed pups had a higher f_R than controls ($p<0.05$ *t* test; Fig. 2*B, middle*). There was no significant difference in the response to R-PIA between preparations from caffeine-treated and control groups (Fig. 4*B*).

A_1 mRNA, DPCPX-binding, and A_1-stimulated GTP$\gamma[^{35}S]$ binding. Adenosine A_1 receptor mRNA was present on E14 in
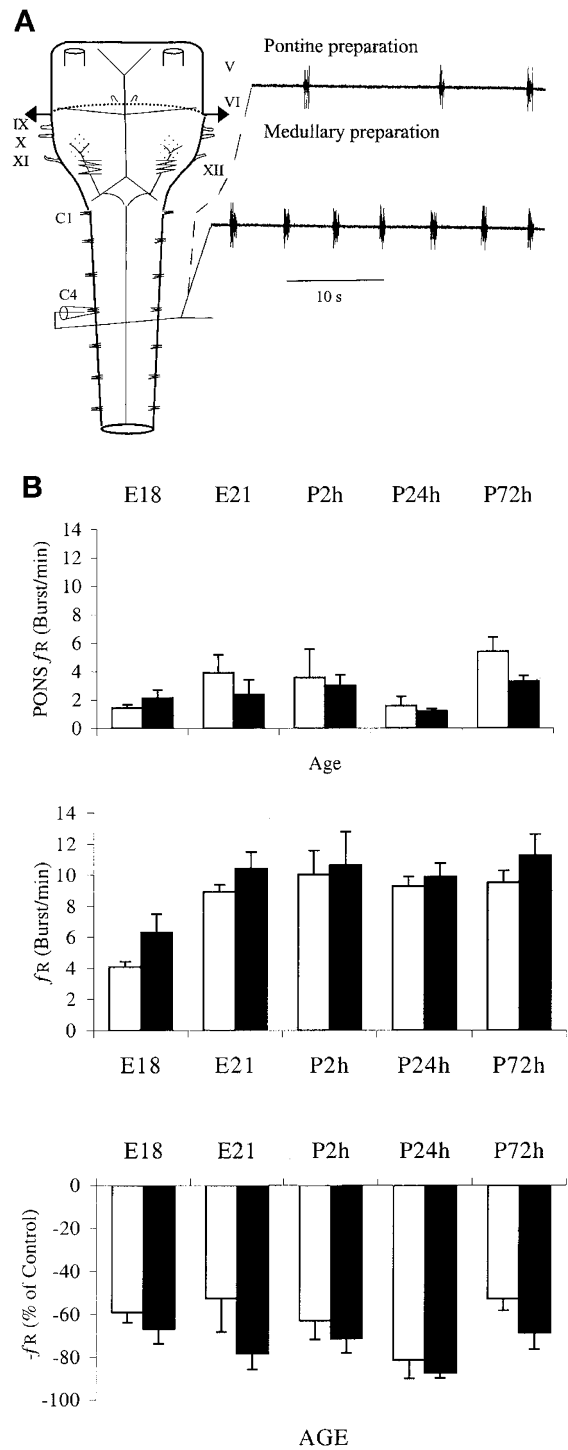
Figure 2. Development of respiratory rhythm generation and its modulation by pons. *A*, schematic illustration of the brain stem–spinal cord preparation is shown on the left side. Dashed line indicates division when removing the pons. Cranial nerves V–XII and ventral roots C1–C4 are shown. On the right side the respiratory activity in a preparation from a 1-d-old postnatal rat before (*upper trace*) and after removal of the pons (*lower trace*). *B*, mean $\pm$ SEM control C4 f_R (bursts/min) in brain stem–spinal cord preparation with pons (*top*) and with pons removed (*middle*), and inhibition exerted by pons on respiratory rhythm generated in medulla oblongata (*bottom*, % of control f_R with pons intact compared with f_R after removal of pons) plotted against age. Both pontine inhibition of respiratory activity and basal f_R were increased in the caffeine-treated group ($p < 0.05$, MANOVA, repeated-measures design). White columns represent control group ($n = 5$–8) and black columns represent caffeine-treated group ($n = 4$–6).
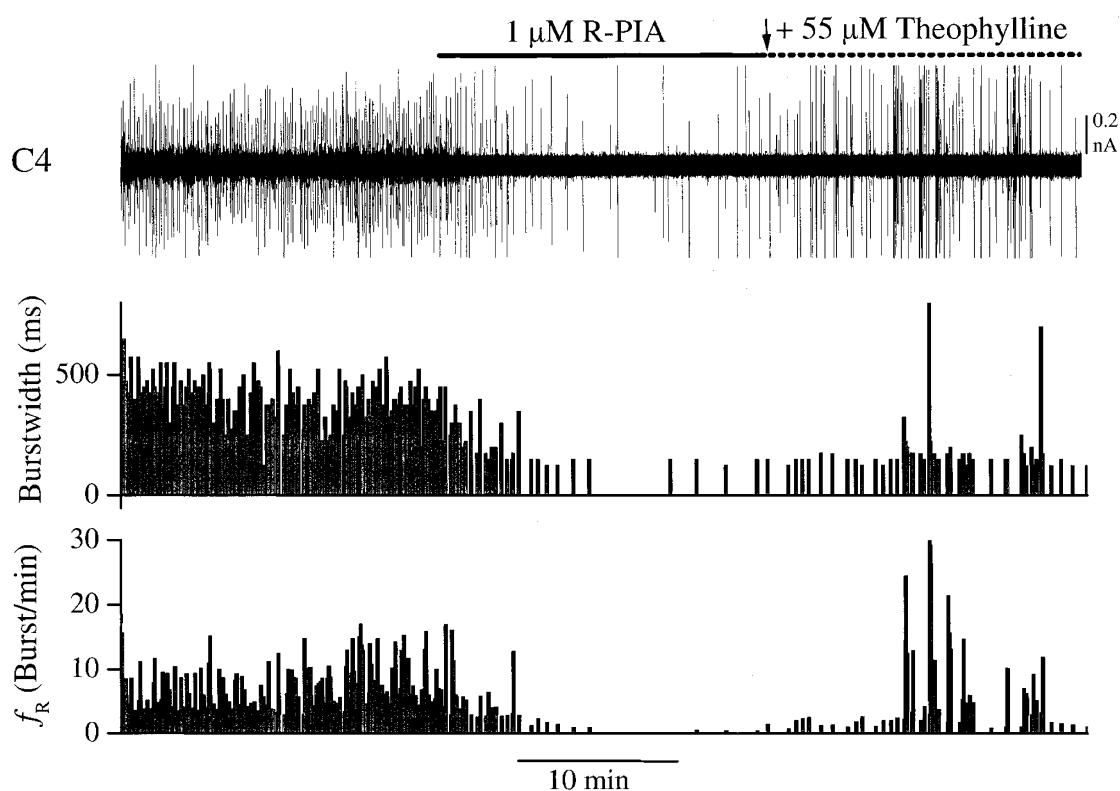
Figure 3. Effects of R-PIA on fetal respiratory activity. Tracings depict respiratory activity recorded from E18 preparation. Note that the effect of R-PIA is pronounced and reversible by theophylline. *Upper trace*, C4 respiratory activity; *middle trace*, burst width (T$_i$) in ms; and *lower trace*, f_R (bursts/min).

low levels in the pontine neuroepithelium. On E21, specific A$_1$ mRNA expression was identified in the pontine gray nucleus (not shown). In pups decapitated shortly after vaginal delivery (2 and 24 h), there were no dramatic changes in adenosine receptors in the brain compared with levels just before birth, but at P3 there was a tendency to a down-regulation of A$_1$ mRNA expression. At P7 A$_1$ mRNA was selectively expressed and DPCPX-binding was enriched in several brain stem nuclei, including the pontine gray nucleus (Fig. 5A). The facial nucleus, lateral reticular nucleus, and the periambiguous area also seemed to have increased binding.

When adenosine A$_1$ receptors were stimulated with CPA, basal GTPγ[^{35}S] binding in the pons and medulla was more than doubled at E18, E21, and P24h ($p < 0.001$, two-way ANOVA), indicating a functional coupling to G-proteins of these receptors already at E18 (Fig. 6). Caffeine-exposed animals had the same levels of A$_1$ mRNA in the pons and medulla as controls, but [^{3}H]DPCPX binding was slightly higher in the caffeine-treated animals than in controls; however, data were only significant when pooled from different age groups. There was an increased [^{3}H]DPCPX binding in the pons when comparing E18–P7 (2–47%, $p = 0.019$) and in the medulla when comparing P24h–P14 (12–37%, $p = 0.018$).

DISCUSSION

The present study shows that whereas there seems to be no dramatic change of basic respiratory rhythm control at birth, an adenosine A$_1$ receptor agonist inhibits the respiratory rhythm generation more markedly before and around birth than 24 h

postnatally. Furthermore, in pups chronically exposed to a low dose of caffeine via maternal intake, the pontine inhibition of brain stem respiratory rhythm is more pronounced compared with that of controls.

Perinatal development of respiratory rhythm. We demonstrate that even though the isolated CPG for breathing continuously matures during perinatal life, no major change in pontine inhibition, f_R, T$_I$, or stability of pattern occurs between E21 and 2 and 24 h after birth. These findings indicate that it is mainly afferent, suprapontine input and changes in the local environment (*i.e.* increased P$_{O_2}$) that alter breathing from a discontinuous to a continuous behavior at birth.

We also show that the pontine inhibition of respiratory centers in the medulla is already present at E18 and does not change between E21 and P3. This inhibition is mediated by noradrenergic activity originating from A5 pontine structures (19). The A5 nucleus appears in the rat on fetal day 17 (31) and our new findings suggest that already at E18, the A5 nucleus modulates medullary respiratory activity. Thus, the pons may be involved in the formation of the early CPG for respiration. This would be in accordance with previous data demonstrating an important role of the pons in the development of medullary respiratory networks and control of breathing (32). We could not demonstrate a change in the pontine inhibition around birth. However, our studies were performed using similar *in vitro* conditions for preparations taken before and after birth. A postnatal change in P$_{O_2}$ would probably decrease the activity of the A5 nucleus *in vivo*, causing less pontine inhibition and thus increasing CPG activity in the medulla oblongata after birth.

A

B
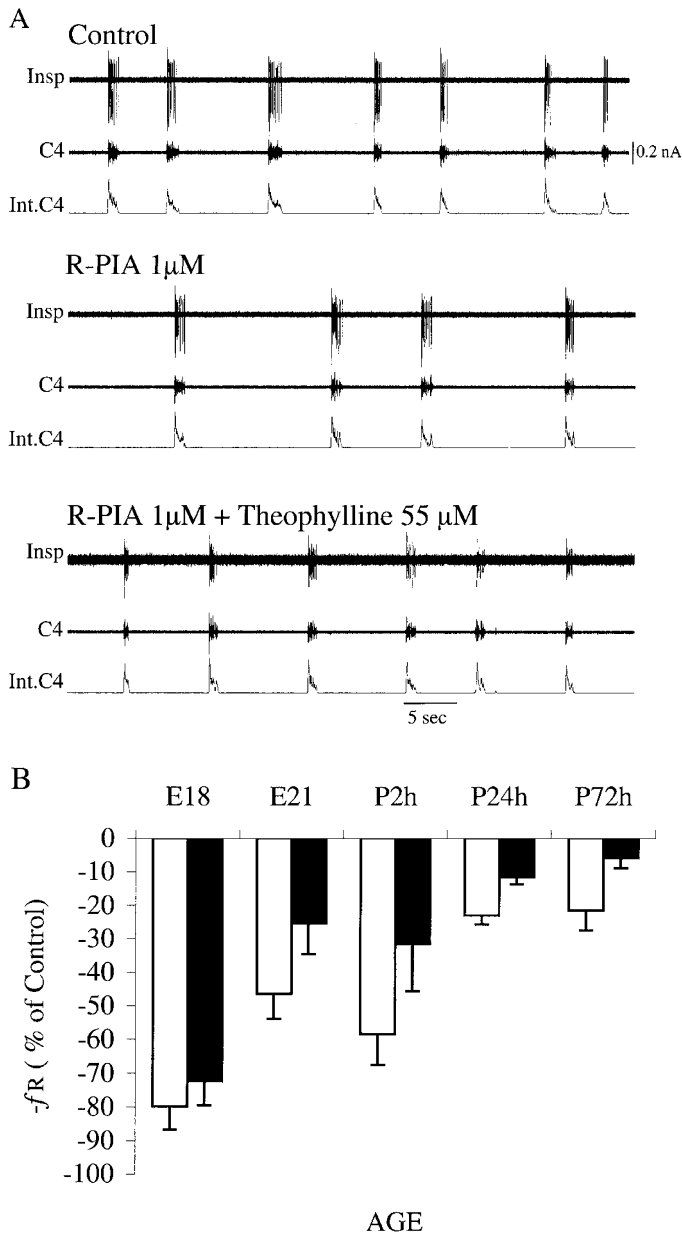
Figure 4. Age-related effects of R-PIA on respiratory activity. *A*, recording from E21 rat and effects of R-PIA (1 μM) and theophylline (55 μM). *Insp*, inspiratory neuron; *C4*, C4 ventral root activity; *Int.C4*, integrated C4. *B*, mean $\pm$ SEM response (% of control) of C4 f_R to R-PIA plotted against age. The effect of R-PIA was related to perinatal age ($p < 0.0001$, MANOVA, repeated-measures design). Within 24 h after birth the respiratory depressant effect of R-PIA is decreased ($p < 0.05$) compared with the effect at E21 and P2 h. No significant difference in the response to R-PIA was found between the control (*white columns*) and caffeine-exposed groups (*black columns*).

***Adenosinergic respiratory depression decrease after birth.***
A reduced ability of adenosine A_1 agonist to decrease respiratory activity occurred already within 24 h postnatally (Fig. 4). This new finding narrows the window when a switch in adenosine's ability to depress breathing occurs. Previous studies in rats (5, 9), rabbits (4), and piglets (33), have reported a similar reduction of adenosinergic effect to occur within the first days or weeks after birth. Immediately after birth, as the partial pressure for oxygen increases, circulating levels of adenosine decrease (3, 34), correlating with decreased extracellular levels in the brain (10), and thus the adenosinergic inhibition of breathing is reduced. Taken together with the present findings, this will contribute to an increased activity of the CPG and establishment of continuous breathing. The changes in f_R were accompanied by a simultaneous change in brain stem respiration-related neuronal activity in all preparations in which recordings were performed. This suggests that adenosine also during the fetal period affects f_R by acting on brain stem respiratory neurons via adenosine A_1 receptors in the medulla oblongata (9).

Caffeine exposure and development of respiratory rhythm.
Here we demonstrate that long-term caffeine intake during gestation and the early postnatal period increases the baseline frequency of C4 respiratory output. Adenosine depresses and adenosine antagonist increases respiration during control conditions (5, 35), suggesting the occurrence of an endogenous adenosinergic tonic activity during the perinatal period. In addition, long-term caffeine intake seems to increase the inhibition exerted by pontine structures *in vitro*. Methylxanthines increase the turnover of noradrenaline (36) and spontaneous electrical activity of noradrenergic neurons. Thus a possible explanation for our findings could be that caffeine, by inducing an increased activity of pontine noradrenergic neurons such as those situated in the A5 area, increases the inhibition from pontine structures to the respiratory centers in the medulla oblongata. This finding is of interest because the pons is involved in respiratory depression during hypoxia (18). We have preliminary *in vivo* data indicating a change in hypoxic respiratory response in caffeine-treated pups, which could agree with the present *in vitro* findings of a change in pontine control of brain stem activity. However, further studies will be needed to elucidate eventual detrimental effects on respiratory control *in vivo*.

A_1 mRNA, DPCPX-binding, and A_1-stimulated GTPγ[^{35}S] binding. A new finding is that A_1 receptor coupling to G-protein occurred prenatally in the brain stem as indicated by A_1-stimulated GTPγ[^{35}S] binding (Fig. 6). This is in contrast to previous studies indicating that the major development in terms of density and coupling to second messenger–forming systems in the cerebral cortex and hippocampus occurs postnatally (37, 38). However, the phylogenetically old brain stem matures earlier than the phylogenetically younger parts of the brain examined in the previous studies. Thus, A_1-receptor coupling to G-proteins occurs prenatally in the brain stem. We found robust A_1 mRNA labeling and DPCPX binding in the pons-medulla toward the end of gestation (E18–E21) and thereafter in accordance with previous studies (39–41). The pontine gray nucleus is connected to cerebellum, and we are not aware of reports indicating a relation to respiratory control. Our finding of increased binding in brain stem nuclei, including the periambiguous area, agrees with a previous study in immature sheep (42), which reported that the highest density of A_1 receptors are found in the rostral ventrolateral medulla, the area that contains the CPG for respiration (43). However, in the present study receptor binding was examined in sagittal slices, and a more precise determination of adenosine A_1 mRNA and A_1 receptor distribution within the brain stem was not per-

A   Receptor-binding   A$_1$-mRNA
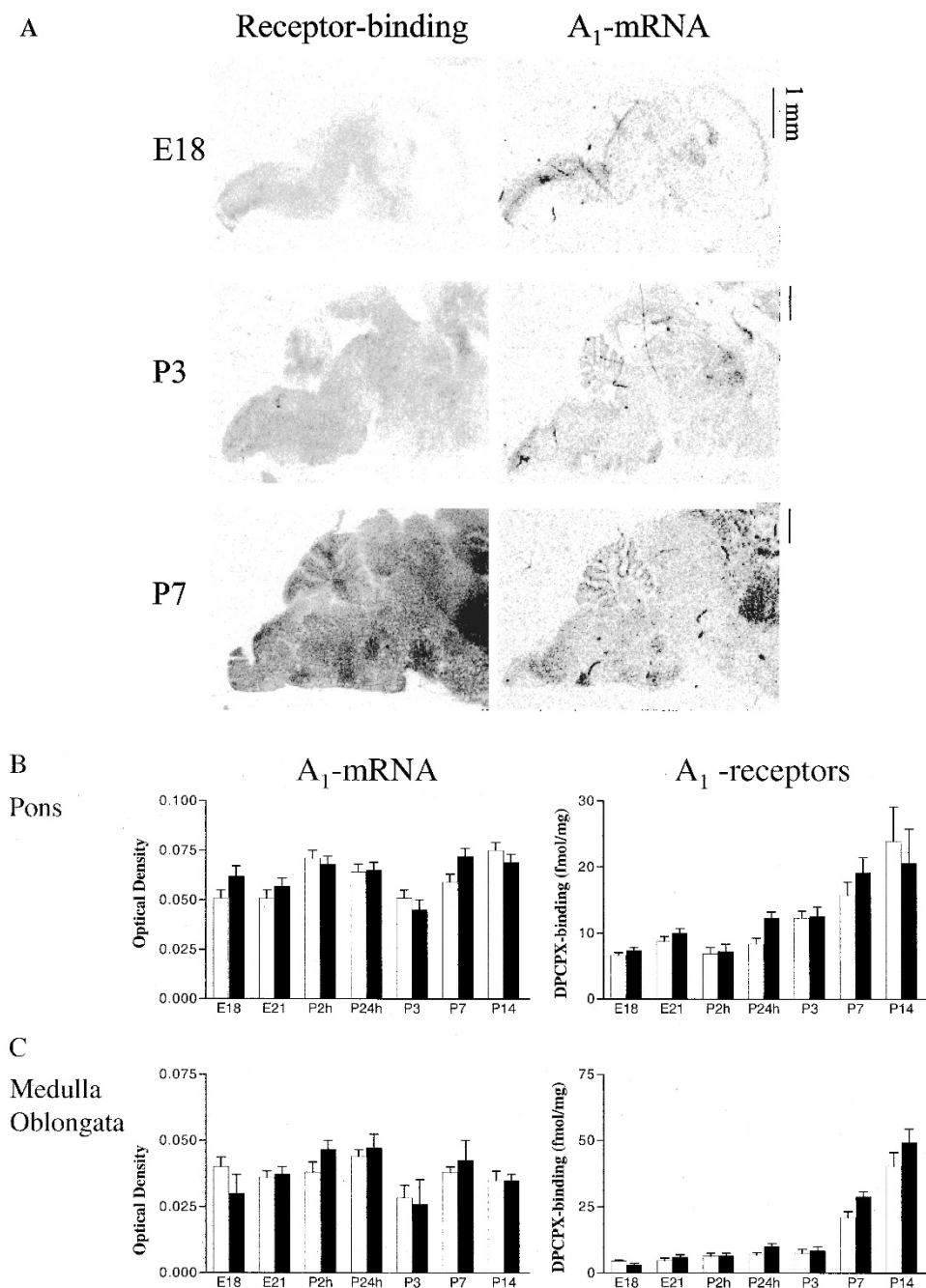
Figure 5. A$_1$-receptor mRNA expression and [^{3}H]DPCPX binding. *A*, film autoradiograms generated from receptor autoradiography (*left side*) and *in situ* hybridization (*right side*) using sagittal sections. Adenosine A$_1$ mRNA expression and A$_1$ receptor binding are present in the brain at E18 (whole brain is shown) and are enriched in several brain stem nuclei at P3 and P7 (caudal parts of the brain are shown). Receptor binding was determined using [^{3}H]DPCPX binding (0.5 nM), and *in situ* hybridization was performed using an oligonucleotide probe. Nonspecific binding was determined and was equal to background. *B*, pre- and early postnatal time course of adenosine A$_1$ mRNA and [^{3}H]DPCPX binding in pons in controls (*white bars*) and low-dose (0.3 g/L) caffeine-treated (*black bars*) rat fetuses and pups. Mean $\pm$ SEM of groups with $n = 6$. *C*, pre- and early postnatal time course of adenosine A$_1$ mRNA and [^{3}H]DPCPX binding in medulla in controls (*white bars*) and low-dose caffeine-treated (*black bars*) rat fetuses and pups. Note that A$_1$ mRNA is generally flat and A$_1$ receptors tend to increase with postnatal age. Mean $\pm$ SEM of groups with $n = 6$.

formed. In pons and medulla there are no major changes in A$_1$ receptor development in caffeine-exposed fetuses and pups, in agreement with our previous report concerning effects of perinatal caffeine exposure to other parts of the brain (44).

One might explain the increased control f_R by a decrease in functional adenosine receptors in the caffeine-treated group. However, if A$_1$ receptors were affected at all by long-term

exposure to low-dose caffeine, they were rather up-regulated. These findings suggest that caffeine affects the adenosine receptors at another level rather than by altering the receptor number. Even though the *in vitro* preparations were superfused with caffeine-free aCSF for a substantial time (104 $\pm$ 7 min) before control measurement was performed, remaining caffeine could explain both the increased f_R and the tendency toward

pons

medulla
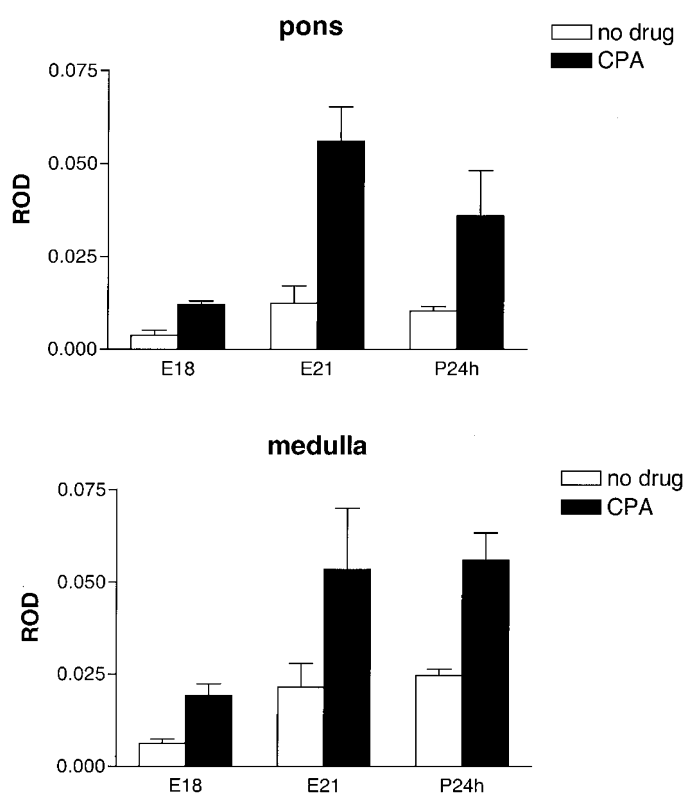
Figure 6. A_1 receptors and functional G-protein coupling. Basal GTPγ[^{35}S] binding (*white bars*) and GTPγ[^{35}S] binding after stimulation with the adenosine A_1 receptor agonist CPA (*black bars*) at E18, E21, and P24h. The increased GTPγ[^{35}S] binding after agonist stimulation indicates a functional coupling of the receptor to the G-protein already at E18 in pons (*top*) and medulla oblongata (*bottom*). Nonspecific binding was determined and was equal to background. *ROD*, relative optical density. Mean ± SEM of groups with $n = 6$.

decreased R-PIA effect in the caffeine group. Alternatively, caffeine affects A_1 receptor signaling downstream of the receptor or induces other types of adaptive changes, as occur in the adult animal in which agonist binding has been shown to increase without detectable changes in A_1 mRNA or antagonist binding (45, 46).

The effect of the adenosine A_1 receptor agonist R-PIA decreased within 24 h after birth. However, there was no corresponding decrease in A_1 receptor binding or A_1-stimulated G-protein coupling 24 h after birth. A decreased binding affinity of A_1 receptor to A_1 agonist has previously been reported to occur with increasing postnatal age (4, 47). This could explain the decreased sensitivity for adenosine A_1 receptor-mediated respiratory depression after birth; however, this was not determined in the present study.

We conclude that respiration is already strongly modulated by adenosinergic action at the level of the medulla oblongata at E18 and that this modulatory action is reduced 24 h after birth. Furthermore, long-term maternal caffeine intake during gestation increases the pontine inhibition of and the activity of medulla oblongata respiratory centers in offspring without changing A_1 receptor mRNA and protein expression.

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