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Disruption of adenosinergic modulation of ventilation at rest and during hypercapnia by neonatal caffeine in young rats: role of adenosine A_1 and A_{2A} receptors

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Montandon G, Kinkead R, Bairam A. Disruption of adenosinergic modulation of ventilation at rest and during hypercapnia by neonatal caffeine in young rats: role of adenosine A_1 and A_{2A} receptors. *Am J Physiol Regul Integr Comp Physiol* 292: R1621–R1631, 2007. First published November 30, 2006; doi:10.1152/ajpregu.00514.2006.—Caffeine is commonly used to treat respiratory instabilities related to prematurity. However, the role of adenosinergic modulation and the potential long-term effects of neonatal caffeine treatment (NCT) on respiratory control are poorly understood. To address these shortcomings, we tested the following hypotheses: *1*) adenosine A_1- and A_{2A}-receptor antagonists modulate respiratory activity at rest and during hypercapnia; *2*) NCT has long-term consequences on adenosinergic modulation of respiratory control. Rat pups received by gavage either caffeine (15 mg/kg) or water (control) once a day from postnatal *days 3* to *12*. At *day 20*, rats received intraperitoneal injection with vehicle, DPCPX (A_1 antagonist, 4 mg/kg), or ZM-241385 (A_{2A} antagonist, 1 mg/kg) before plethysmographic measurements of resting ventilation, hypercapnic ventilatory response (5% CO_2), and occurrence of apneas in freely behaving rats. In controls, data show that A_{2A}, but not A_1, antagonist decreased resting ventilation by 31% ($P = 0.003$). A_1 antagonist increased the hypercapnic response by 60% ($P < 0.001$), whereas A_{2A} antagonist increased the hypercapnic response by 42% ($P = 0.033$). In NCT rats, A_1 antagonist increased resting ventilation by 27% ($P = 0.02$), but the increase of the hypercapnic response was blunted compared with controls. A_1 antagonist enhanced the occurrence of spontaneous apneas in NCT rats only ($P = 0.005$). Finally, A_{2A} antagonist injected in NCT rats had no effect on ventilation. These data show that hypercapnia activates adenosinergic pathways, which attenuate responsiveness (and/or sensitivity) to CO_2 via A_1 receptors. NCT elicits developmental plasticity of adenosinergic modulation, since neonatal caffeine persistently decreases ventilatory sensitivity to adenosine blockers.

control of breathing; carbon dioxide chemosensitivity; ventilation; plasticity; hypercapnic ventilatory response

AT BIRTH, THE RESPIRATORY control system of mammals is immature, and respiratory instabilities, such as apneas, are frequently observed (1, 43). Caffeine, an adenosine-receptor blocker, is commonly used as a respiratory stimulant in infants born prematurely to treat respiratory instabilities. However, the use of caffeine during a critical period of development is always a matter of concern given the potential impact on central nervous system maturation and respiratory functions (19). In utero chronic caffeine administration induced changes in respiratory activity (5, 28) and in Fos expression in brain stem respiratory nuclei of newborn rats (5). Exposure to caffeine during the early postnatal period enhanced the ventilatory response to hypercapnia in 20-day-old young male rats (47) and modified the distribution of adenosine A_1 receptors in respiratory nuclei of the newborn rat brain stem (23). Young rats showed the largest sensitivity to neonatal caffeine treatment (NCT), and from a developmental standpoint it has been proposed that this age group is comparable to infants (10) for which respiratory instabilities, such as apneas (42), can be observed.

Adenosine A_1 and A_{2A} receptors modulate ventilatory control in newborn or fetal sheep (38, 39). Adenosine A_1 receptors modulated breathing during normoxia in fetal sheep (39), whereas adenosine A_{2A} receptors regulate ventilation at the level of arterial peripheral carotid body in fetal sheep (37) and adenosine A_{2A}-receptor antagonist reversed the hypoxic ventilatory decline in newborn lambs (38). However, the roles of adenosine A_1 and A_{2A} receptors on ventilation at rest and on the ventilatory response to hypercapnia are not fully understood in young rats. With that in mind, we first administered specific adenosine A_1- and A_{2A}-receptor antagonists to identify the specific roles of these receptors on ventilation at rest, during acute exposure to hypercapnia (as it occurs during asphyxia), and on the occurrence of apneas in freely behaving young male rats (20 days old).

Caffeine has different affinities for adenosine A_1 or A_{2A} receptors (22), and selective activation of each receptor subtype has different functional effects (27, 31, 34, 39). Furthermore, adenosine A_1 and A_{2A} receptors are differently distributed: A_1 receptors are widely spread in the nervous system (58, 59), whereas A_{2A} receptors are restricted to the striatum, the nucleus accumbens (61), the carotid bodies (63), and several distinct brain stem nuclei (61, 68). From these observations, the second aim of this study was to test the hypothesis that NCT persistently and distinctly modifies the effect that each adenosine-receptor subtype exerts on ventilatory activity at rest and during acute hypercapnic exposure.

Our results show that, in control rats, adenosine A_1-receptor inactivation increased the hypercapnic ventilatory response, indicating a role for adenosine A_1 receptors on CO_2 chemosensitivity. However, adenosine A_{2A}-receptor inactivation had a small effect on the hypercapnic response, although resting minute ventilation ($\dot{V}E$) had decreased. Caffeine exposure during early life elicits significant plasticity of adenosinergic modulation of the respiratory control system because NCT decreased the effect of A_1 antagonist and increased the occurrence of spontaneous apneas when adenosine A_1 receptors are inactivated by a specific antagonist. Finally, NCT blunted the decrease of ventilation due to A_{2A}-receptor antagonist observed in control rats.

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MATERIALS AND METHODS

Animal and Housing Conditions

The study was performed on 75 young (postnatal *day 20*) Sprague-Dawley rats. All rats were born in our animal care facility. Dams and males were obtained from Charles River Canada (St. Constant, QC, Canada). Rats were supplied with food and water ad libidum and maintained in standard laboratory conditions (21°C, 12:12-h dark-light cycle, with lights on at 08:00 and off at 20:00). The Laval University Animal Care Committee approved the experimental procedures, and all protocols were in accordance with the guidelines detailed by the Canadian Council on Animal Care.

Mating and NCT

On average, dams delivered 10 ± 2 pups. Although only males were used in this study, the litter size was kept constant (whenever possible) at 12 pups by keeping females. There were 6 ± 2 male pups in each litter. Administration of caffeine was performed according to our protocol described previously (47). Briefly, caffeine was administered by gavage each day from postnatal *days 3* to *12* with 15 mg/kg caffeine citrate (Sabex, Boucherville, QC, Canada) in a volume of 0.05 ml/10 g body wt. The control group was subjected to the same treatment but received the same volume of water. This caffeine dose results in a plasmatic caffeine level of 13 ± 3 mg/l (47), which is comparable to the level achieved in the clinic when caffeine is administered therapeutically to newborns (2, 4).

Adenosine Receptor Antagonists

The specific adenosine A_1-receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 4 mg/kg; Tocris, Ballwin, MO) and the adenosine A_{2A}-receptor antagonist 4-{2-[7-amino-2-(53)triazolo-(2,3-a)(1, 3, 5)triazin-5-yl-amino]ethyl}-phenol (ZM-241385, 1 mg/kg; Tocris) were used according to protocols and doses from other studies on rats (15, 57). Each drug was freshly prepared on the day of the experiment: it was first dissolved in DMSO (Sigma-Aldrich, Oakville, ON, Canada), and then a solution of polyether of castor oil and ethylene oxide (Cremophor EL; Sigma) was added to prevent drug precipitation. Before administration, each drug was diluted with saline to a final concentration of 5% DMSO and 5% Cremophor EL and given intraperitoneally in a volume of 0.5 ml/100 g body wt.

Experimental Groups

Two groups were used in this study: one received water (control) and the other received caffeine (NCT) from postnatal *days 3* to *12*. Each group was divided into three subgroups: vehicle, A_1 antagonist (DPCPX), and A_{2A} antagonist (ZM-241385). Vehicle solution was made of 5% DMSO and 5% Cremophor EL in saline. Vehicle subgroups in both control and NCT animals were used to ensure that vehicle and stress from injections had no effect on respiratory measurements. Ventilatory activity was measured at 20 days of age (young rats) for each subgroup.

Respiratory and Metabolic Measurements

Respiratory assessments. Measurements of breathing frequency (f_R), tidal volume (V_T), and inspiratory duration (T_I) in unrestrained rats were obtained by whole body, flow-through plethysmography (model PLY3223; Buxco Electronics, Sharon, CT) as previously described in details (36, 47). An apnea was identified according to previous criteria, which defines apnea as an absence of flow for a duration of two normal breathing cycles (46), corresponding to an interruption of at least 1 s in young rats (47). Two types of apneic pauses were collected: spontaneous and postsigh apneas. The spontaneous apnea was identified when an interruption of flow suddenly occurred during inspiration, whereas the postsigh apnea was identified as an interruption of flow preceded by a breath with an amplitude that exceeded 2× resting V_T (47). Rectal temperature was measured twice during experiments: at the beginning of resting measurements and at the end of hypercapnia. Barometric pressure, chamber temperature, humidity, and body temperature were used to express V_T in milliliters (BTPS) per 100 g body wt according to standard equations (13, 52). Because no differences of rectal temperature were observed between rest and hypercapnia, we used rectal temperature measured at the end of hypercapnia to correct V_T during hypercapnia. $\dot{V}_E$ was defined as the product of V_T and f_R.

Metabolic assessments. O_2 and CO_2 levels of the gas mixture flowing in and out of the chamber were measured with an oxygen analyzer (model S-3A; Ametek, Pittsburgh, PA) and a carbon dioxide analyzer (model CD-3A; Ametek). These values were used to calculate oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) according to the Fick principle, $([O_2]_{in} - [O_2]_{out}) \times$ flow and $([CO_2]_{out} - [CO_2]_{in}) \times$ flow, respectively (where brackets indicate concentration), which is commonly used in an open system (51).

Respiratory Protocol

Each rat was introduced into the plethysmographic chamber about 30 min before recordings for acclimation. Resting ventilatory and metabolic measurements were made when the rat was quiet but awake and breathing room air by data-acquisition software (IOX; EMKA Technologies, Falls Church, VA). After 5 min of normoxic normocapnic measurements (rest), a hypercapnic gas mixture (inspired CO_2 fraction = 0.05; inspired O_2 fraction = 0.20) was delivered to the chamber for 20 min. Each experiment was performed between 0900 and 1300, and then the rat was killed by CO_2 asphyxia followed by decapitation according to standard procedures. Breaths were detected by the data-acquisition software, and acceptation/rejection of individual breaths was performed automatically. The software's default values for ventilatory parameters are usually adequate to reject signals related to movement artifacts. However, sniffing-related signals were excluded by setting the T_I rejection threshold above 0.12 s (47).

Data and Statistical Analyses

Average values of ventilatory variables were obtained on a minute by minute basis using data analysis software (DataAnalyst; EMKA). We calculated the mean values of five consecutive minutes at rest (Fig. 1). Also, at rest, individual data are presented to assess homogeneity of breath distribution among rats (Fig. 2). Group data (n = 12, 5 min of resting breathing) of breath duration (0.2–0.7 s) vs. V_T (0–3 ml/100 g) are presented for ~2,000 breaths for each group. Individual breaths are converted into a two-dimensional histogram (bin width, 0.02 s and 0.05 ml/100 g) and plotted as density maps for each group and each drug. Colors represent the relative number of breaths in a given bin as percentages of breaths in the maximum bin. Flattened comparisons of all bins with densities >40% are also presented. This representation, inspired by a previous study (25), allowed us to distinguish individual distribution of breaths for each group, information not given by the mean.

The ventilatory response to hypercapnia for selected variables was expressed as a percent change of hypercapnic value relative to resting average. A value of 0% signifies that there was no change due to hypercapnia, whereas a value of 100% indicates a twofold increase compared with resting value. A 4-min average was taken every 4 min of hypercapnic exposure up to 20 min to express the time course of hypercapnic ventilatory response. Finally, mean apnea indexes of spontaneous and postsigh apneas were counted visually at rest according to the criteria described previously and were expressed as number of apnea per 10 min.

To test whether antagonists have a significant influence on resting ventilatory values, hypercapnic ventilatory responses, or apnea indexes, we used a one-way ANOVA (fixed factor: antagonist; JMP 5.1,

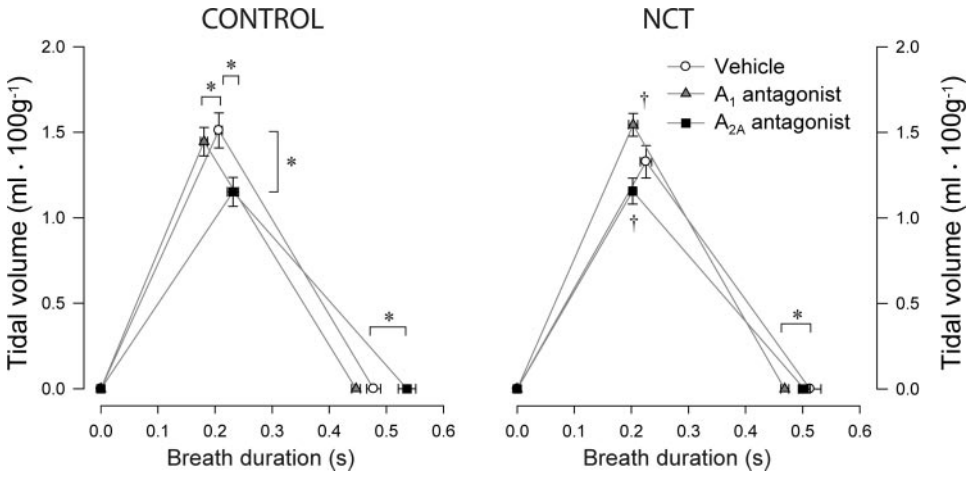

Fig. 1. Mean respiratory cycles at rest after injection of either vehicle, adenosine A_1-receptor antagonist (DPCPX), or adenosine A_{2A}-receptor antagonist (ZM-241385) in control and neonatal caffeine treatment (NCT) groups of male young rats. Values are means ± SE of vehicle (○) and A_1 (gray triangles) and A_{2A} (■) antagonists. *Significantly different from vehicle ($P < 0.05$). †Significantly different from respective control duration.

SAS Institute, Cary, NC) for control or NCT animals (P values of ANOVA are presented in the text). Dunnett's test was used as a post hoc test to compare the mean of each antagonist with the mean of the vehicle group. To determine whether caffeine treatment has an effect on resting ventilatory values, hypercapnic ventilatory responses, or apnea indexes for each antagonist, we used one-way ANOVA with caffeine treatment as a fixed factor. Finally, to test whether caffeine treatment has an effect on antagonist influence, we used two-way ANOVA (the fixed factors were antagonists and treatments) followed by post hoc analysis with least-significant mean difference Student's t-tests (JMP 5.1). This allowed us to determine whether there was an interaction between caffeine treatment and the influence of adenosine antagonists. Data were considered statistically different when $P < 0.05$ and are expressed as means ± SE.

RESULTS

Ventilation at Rest

Inactivation of adenosine A_{2A}, but not A_1, receptors decreases $\dot{V}_E$ at rest in control rats. The A_1 antagonist did not change $\dot{V}_E$ compared with vehicle (Table 1) but decreased T_I by 13% ($P = 0.03$; Fig. 1). However, the A_{2A} antagonist decreased resting $\dot{V}_E$ by 31% ($P = 0.003$) compared with vehicle rats (Table 1). This decrease was due to a 10% lower f_R ($P = 0.0006$; Fig. 1) and 24% lower V_T ($P = 0.02$; Fig. 1). A_1 antagonist had no significant effect on $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, even though body temperature was increased by 0.6°C ($P = 0.0004$). Injection with A_1 antagonist had no significant effect on $\dot{V}_E$-to-$\dot{V}_{O_2}$ and $\dot{V}_E$-to-$\dot{V}_{CO_2}$ ratios (Table 1). Moreover, no metabolic changes were observed in A_{2A} antagonist compared with vehicle (Table 1). Both convective requirement ratios were decreased by the A_{2A} antagonist compared with vehicle-injected rats ($\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$, $P = 0.004$ and $P = 0.009$, respectively; Table 1).

Inactivation of adenosine A_1, but not A_{2A}, receptors increases $\dot{V}_E$ at rest in NCT rats. In NCT rats, A_1 antagonist increased $\dot{V}_E$ by 27% ($P = 0.02$; Table 1), owing to a 9% increase in f_R ($P = 0.04$). Density maps show that NCT rats injected with A_1 antagonist caused a greater V_T for a population of breaths (Fig. 2*B*; *right*), an effect not observed when comparing mean values. Unlike controls, the A_{2A} antagonist had no effect on $\dot{V}_E$ measured in NCT rats. Similar results were observed for f_R and V_T (Fig. 1, *right*). Moreover, no changes were observed for $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ after A_1 antagonist injection (Table 1). However, A_1 antagonist increased $\dot{V}_E/\dot{V}_{O_2}$ by 27% ($P = 0.03$) and body temperature by 0.4°C ($P = 0.005$). A_{2A} antagonist increased body temperature by 0.5°C ($P = 0.008$; Table 1).

Impact of NCT on the ventilatory effects at rest of adenosine A_1- and A_{2A}-receptor antagonists. In vehicle-injected rats, no significant differences were observed between control and NCT rats at rest for any of the respiratory variables measured. Similar resting values were observed in our previous study for control and NCT rats of the same age group (47). However, detailed analysis of the resting breathing pattern with two-dimensional density maps showed the presence of two distinctive spots in Fig. 2*A*, *right*, indicating that, although control rats had an homogenous breathing activity, NCT altered breathing pattern in a way that resulted in two types of breaths: low- and high-duration breaths (L and H arrows, respectively, in Fig. 2, *A* and *D*). Thus NCT rats presented high-respiratory (~160 min^{-1}) and low-respiratory frequencies (~115 min^{-1}) (low- and high-breath durations, respectively). Finally, metabolic data showed that NCT decreased $\dot{V}_E/\dot{V}_{O_2}$ in vehicle rats by 25% ($P = 0.02$; Table 1).

ANOVAs of treatments (control vs. NCT) × antagonists (vehicle vs. A_1 or A_{2A}) allowed us to evaluate the impact of NCT on adenosine-receptor modulation of ventilation at rest. These tests show that, despite suggestive trends, NCT had no significant impact on ventilatory ($\dot{V}_E$) effects of A_1 and A_{2A} antagonist ($P = 0.12$ and $P = 0.11$, respectively). However, the effect of A_{2A} antagonist on f_R and T_I changed according to the treatment ($P = 0.004$ and $P = 0.038$, respectively). NCT blunted the decrease of f_R due to A_{2A} antagonist (increase of breath duration in Fig. 1) observed in control rats. No changes due to NCT were observed in metabolic data after antagonist injections.

Hypercapnic Ventilatory Response

Inactivation of either adenosine A_1 or A_{2A} receptors increases the hypercapnic ventilatory response of control rats. In control rats, ventilatory responses expressed as a percent change from resting value are shown in Fig. 3. Adenosine A_1 antagonist increased $\dot{V}_E$ response by at least 60%. This effect was especially noticeable immediately during the first 10 min of the onset of hypercapnia (Fig. 4*A*, *left*), when a strong f_R increase was observed (Fig. 4*B*). V_T response was increased by the A_1 antagonist; however, unlike the f_R response, this effect became more apparent

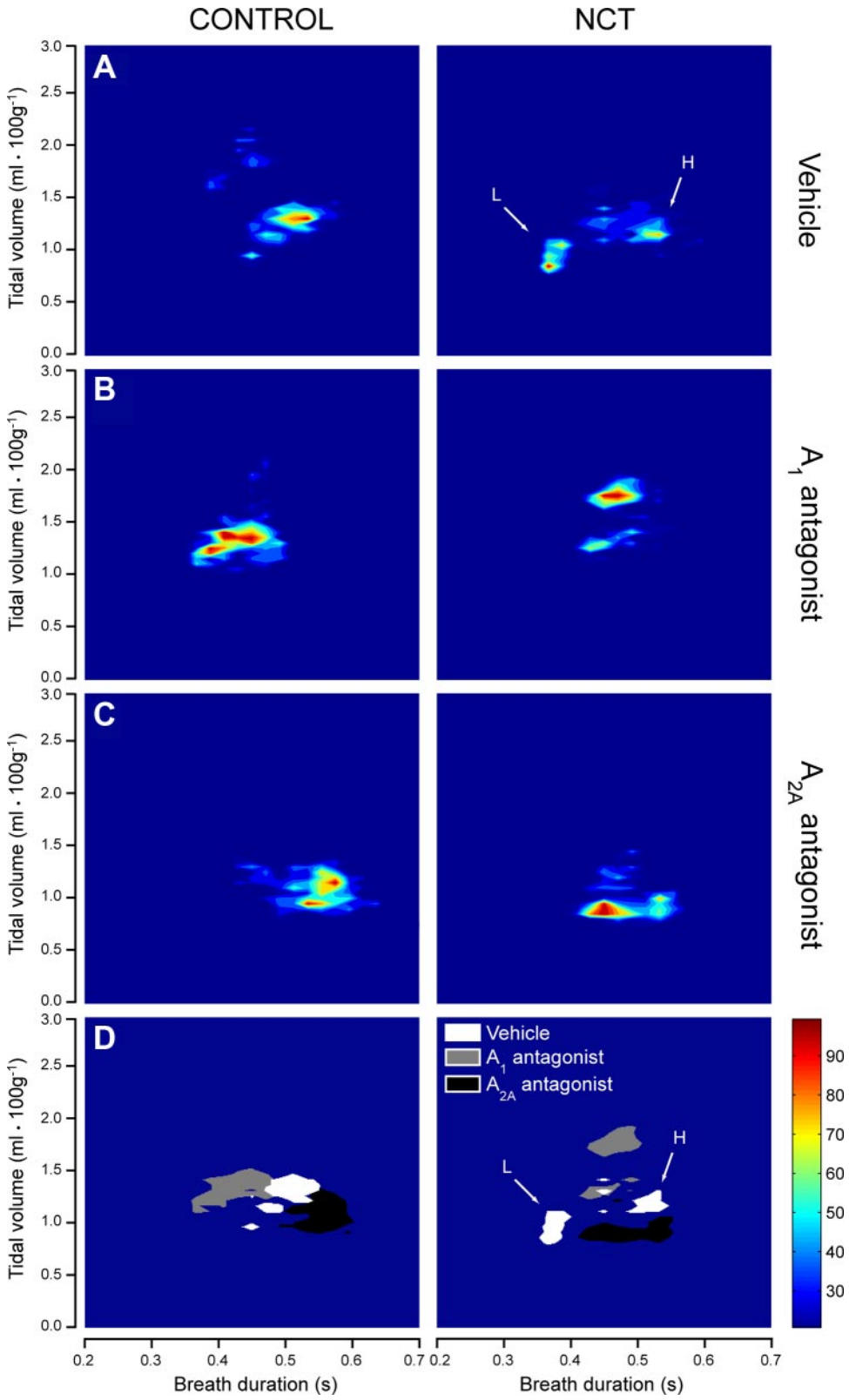

Fig. 2. Density map of breaths at rest after injections with either vehicle or A_1-receptor (DPCPX) or A_{2A}-receptor (ZM-241385) antagonists in control and NCT rats. Each graph represents group data (n = 12, 5 min of resting breathing) of total breath duration (0.2–0.7 s) vs. tidal volume (0–3 ml/100 g) for ~2,000 breaths. Individual breaths were converted into a 2-dimensional histogram (bin width, 0.02 s and 0.05 ml/100 g) and plotted as density maps. In *A* (vehicle), *B* (A_1 antagonist), and *C* (A_{2A} antagonist), colors represent the relative number of breath in a given bin as percentages of breaths in the maximum bin for control (*left*) and NCT (*right*) rats. *D*: flattened comparison between vehicle (white), A_1 antagonist (grey), and A_{2A} antagonist (black) rats of all bins with densities >40%. Arrows show low-duration (L) and high-duration (H) breaths.

by the late phase of the response (*minutes 12–20*; Fig. 4*C*). A_{2A} antagonist caused a more modest increase of the $\dot{V}_E$ response to hypercapnia (by 42%; Fig. 4*A*). Again, this was mainly due to a strong response of f_R (Fig. 4*B*); however, unlike A_1 rats, this effect was significant only during the late phase of the response. Finally, A_{2A} antagonist had no significant effect on the V_T response (Fig. 4*C*).

NCT reduces the effects of adenosine A_1- and A_{2A}-receptor inactivation on the hypercapnic ventilatory response. In NCT rats, injection of the A_1 antagonist increased $\dot{V}_E$ response to

Table 1. *Resting ventilatory, metabolic, and body weight data in control and NCT young male rats injected with vehicle, A_1-receptor (DPCPX), and A_{2A}-receptor antagonist (ZM-241385)*

	Control Rats			NCT Rats		
	Vehicle (n = 14)	A_1 Antagonist (n = 12)	A_{2A} Antagonist (n = 12)	Vehicle (n = 13)	A_1 Antagonist (n = 12)	A_{2A} Antagonist (n = 12)
$\dot{V}_E$ (BTPS), ml·min^{-1}·100 g^{-1}	194±14	198±11	133±12*	160±13	203±9*	141±9
$\dot{V}_{O_2}$ (STPD), ml·min^{-1}·100 g^{-1}	3.58±0.18	4.06±0.27	3.75±0.18	3.89±0.17	3.95±0.18	3.36±0.23
$\dot{V}_{CO_2}$ (STPD), ml·min^{-1}·100 g^{-1}	3.52±0.19	3.48±0.19	3.43±0.19	3.51±0.19	3.68±0.16	3.60±0.21
$\dot{V}_E/\dot{V}_{O_2}$	55.6±4.6	51.3±4.4	36.3±3.5*	41.5±3.4†	52.7±3.3*	44.9±5.1
$\dot{V}_E/\dot{V}_{CO_2}$	56.8±5.0	59.1±4.9	39.3±3.1*	46.3±3.7	56.5±3.5	41.7±5.0
T_{rect}, °C	36.5±0.1	37.1±0.1*	36.8±0.1	36.4±0.1	36.8±2*	36.9±0.1*
Body weight, g	56±2	54±1	56±2	53±3	50±1	53±2

Values are means ± SE; n = no. of rats. $\dot{V}_E$, minute ventilation; $\dot{V}_{O_2}$, oxygen consumption; $\dot{V}_{CO_2}$, carbon dioxide production; $\dot{V}_E/\dot{V}_{O_2}$, oxygen convection ratio; $\dot{V}_E/\dot{V}_{CO_2}$, carbon dioxide convection ratio; T_{rect}, rectal temperature. *$P < 0.05$ vs. vehicle; †$P = 0.05$ vs. control.

hypercapnia by at least 30% (Fig. 4*A*, *right*). As for controls, this increase was due to a strong enhancement of the f_R response (Fig. 4*B*). Unlike control rats, however, A_{2A} antagonist had no net effect on any of the responses measured in NCT rats. Comparisons between enhancements caused by each antagonists showed that $\dot{V}_E$ and f_R responses from A_1 antagonist-treated rats were stronger than those from the A_{2A} antagonist group ($P < 0.024$). Comparisons between control and NCT vehicle rats showed that NCT increased the $\dot{V}_E$ response by at least 27% at the end of hypercapnia (*minutes 16* and *20*, $P = 0.02$ and $P = 0.048$; Fig. 4*A*) as observed previously (47). However, A_1 antagonist caused a smaller enhancement of the $\dot{V}_E$ response to CO_2 in NCT rats than in control rats (*minutes 8* and *12*, $P = 0.007$ and $P = 0.03$, respectively; Fig. 4*A*). These differences were mainly due to a decreased V_T response in NCT rats (*minutes 8* and *12*, $P = 0.02$ and $P = 0.006$; Fig. 4*C*). By comparing A_{2A} antagonist-related changes and NCT influences, we observed a decrease of the $\dot{V}_E$ response in NCT rats vs. controls (*minutes 4*, *16*, and *20*, $P = 0.049$, $P = 0.02$, and $P = 0.01$; Fig. 4*A*), due in part to a decrease of the f_R response (*minute 20*, $P = 0.02$; Fig. 4*B*) in the NCT rats. ANOVAs of antagonists (A_1 and A_2 antagonist groups) × treatments (control and NCT) demonstrated that NCT diminished in a stronger manner the V_T response of A_1 antagonist-treated rats than of A_{2A} antagonist-treated rats (*minutes 8* and *12*, $P = 0.04$ and $P = 0.02$; Fig. 4*C*).

Occurrence of Apneas

Inactivation of adenosine A_1, but not A_{2A}, receptors increases spontaneous apneas only in NCT rats. Figure 5 compares the mean data of apnea occurrence between control and NCT groups at rest. NCT did not change the occurrence of spontaneous apneas in vehicle-injected rats. However, the occurrence of spontaneous apneas was altered by adenosine antagonist in a way that varied according to treatment. In control rats, no differences were observed between vehicle, A_1, and A_{2A} antagonist rats. In NCT rats, however, A_1 antagonist increased spontaneous apneas by 165% ($P = 0.029$). ANOVAs demonstrated that the A_1 antagonist effect was specific to NCT rats ($P = 0.005$).

Inactivation of adenosine A_1, but not A_{2A}, receptors decreases postsigh apneas only in NCT rats. In vehicle-treated rats, we showed that NCT did not change the occurrence of postsigh apneas (Fig. 5). Neither the A_1 nor A_{2A} antagonist affected postsigh apnea occurrence when injected in control rats. In NCT rats, however, A_1 antagonist decreased postsigh apnea index by 44% ($P = 0.003$). This effect was observed even though A_1 antagonist increased the total number of sighs by 52% ($P = 0.02$) at rest in NCT rats. Conversely, A_{2A} antagonist had no effect on the occurrence of postsigh apneas.

DISCUSSION

By administrating specific adenosine-receptor antagonists in control animals, we revealed the role of adenosinergic neurotransmission in respiratory control at rest and during hypercapnic exposure in young (20 days old), freely behaving rats (Table 2). In control rats, adenosine A_1 antagonist increased the hypercapnic ventilatory response but had no effect on ventilation at rest. However, although adenosine A_{2A} antagonist enhanced the hypercapnic ventilatory response only slightly, it caused a strong decrease of ventilation at rest. These results suggest that, in control rats, adenosine exerts tonic excitation of resting ventilation via A_{2A}-receptor activation. During hypercapnia, however, adenosinergic modulation helps attenuate the hyperventilatory response. The fact that this effect involves a different receptor subtype (A_1) than under resting conditions (A_{2A}) suggests that adenosine acts on a different component of the respiratory control system under each condition. Moreover, NCT elicits significant plasticity of adenosinergic modulation of respiratory control because it decreased the facilitating effect of A_1 antagonist on the hypercapnic ventilatory response and blunted the diminution of resting ventilation due to A_{2A} antagonist. In that regard, NCT also increased the occurrence of spontaneous apneas when adenosine A_1 receptors were inactivated by a specific antagonist. Thus caffeine exposure during early life elicits significant plasticity of adenosinergic modulation of the respiratory control system of young rats because the overall neuromodulatory influences of adenosine (both at rest and during hypercapnic challenge) suggest that caffeine alters the expression and sensitivity of each receptor subtype differently.

Critique of Methods

Plethysmography. The accuracy of V_T measurements obtained with whole body plethysmography is the subject of much debate (16, 32). In the present study, obtaining an accurate V_T measurement can be enhanced, since, in small animals, high airway resistance can lead to pressure changes

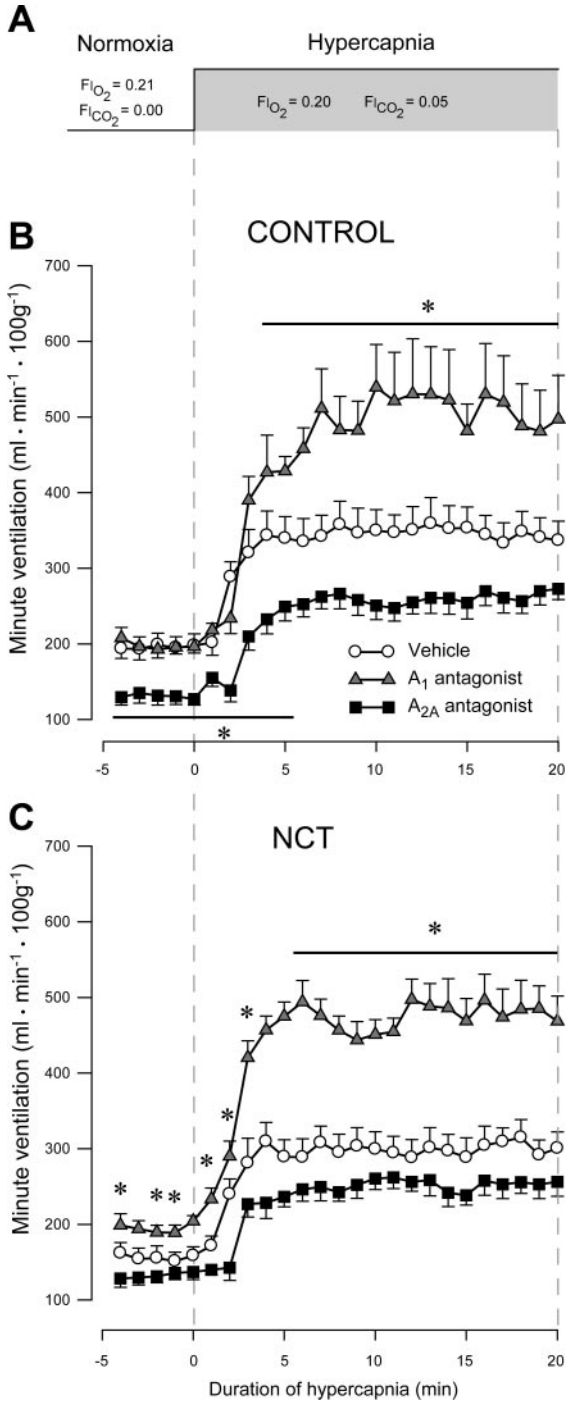

Fig. 3. Time courses of minute ventilation during hypercapnia after injection with either vehicle or A_1-receptor (DPCPX) or A_{2A}-receptor (ZM-241385) antagonists in control and NCT male young rats. *A*: sequence of hypercapnia. *B* and *C*: control and NCT rats, respectively. Values are means ± SE of vehicle (○), A_1 antagonist (gray triangles), and A_{2A} antagonist (■). FI_{O_2} and FI_{CO_2}, inspired O_2 and CO_2 fraction, respectively. *Significantly different ($P < 0.05$) compared with respective vehicle value.

associated with compression and rarefaction of gas. However, this method remains the best approach to measure ventilatory variables (including VT) in awake, freely behaving animals. To minimize errors due to the correction of VT, the hypercapnic ventilatory response was expressed in percent change from baseline value (32). Also, it cannot be ruled out that the VT decrease observed after A_{2A} antagonist injection was due to a change in airway resistance. Plethysmography does not allow us to distinguish a change in VT due to airway resistance variation from a change in the neural command of breathing. However, although activation of adenosine A_1 receptors has a bronchoconstrictor effect, A_{2A}-receptor activation has no impact on airway resistance (60).

Injection of drugs. Intraperitoneal injection of antagonist could pose several problems. Little is known about the pharmacodynamics of DPCPX and ZM-241385; however, based on previous studies, it would appear that DPCPX reached the brain at least 30 min postinjection and the concentration is five times lower in the brain than in the serum (20). Although ZM-241385 pharmacodynamics are unknown, the dose of antagonist used in the present study is sufficient to alter cognitive capacities (55–57). However, comparison of the effects of each antagonist must be done very carefully, since comparisons of their pharmacodynamics have not been performed.

Other adenosine-receptor subtypes. The possibility that caffeine, as well as the A_{2A} antagonist, also blocks adenosine A_{2B} and A_3 receptors cannot be ruled out. Indeed, caffeine has a good affinity for A_{2B} receptors but is poorly selective for A_3 receptors (21). ZM-241385 is, however, highly selective for A_{2B} receptors (8). The roles of adenosine A_{2B} and A_3 receptors in respiratory control are not clear. Activation of adenosine A_{2B} receptors in the carotid bodies exerted excitatory effects on carotid body chemoreceptors output (12), whereas no roles have been demonstrated for A_3 receptors.

Role of Adenosine Receptors in Control of Breathing in Awake Young Rats

Ventilation at rest. In our study, injection of A_1 antagonist did not modify ventilation at rest in male young rats (Table 2). These results contrast with those obtained in nonanesthetized fetal sheep (39) and in in vitro preparations of newborn rat brain stem (65) in which a specific adenosine A_1-receptor antagonist increases, respectively, f_R and the frequency of inspiratory neurons. Furthermore, adenosine A_1-receptor agonist decreased $\dot{V}_E$ in anesthetized (40) and conscious adult rats (40, 66). These discrepancies may be related to the fact that we used 20-day-old rats instead of newborns or adults because specific binding of the adenosine analog is age dependent in rats (26).

In the present study, the adenosine A_{2A}-receptor antagonist decreased resting $\dot{V}_E$, owing to lower f_R and VT. Previous studies that used anesthetized, immature rats (44) or en bloc brain stem preparations from newborn rats (29) yielded opposite results, as central activation of adenosine A_{2A} receptors by selective agonists decreased breathing. Vagotomy, the absence of peripheral chemosensory input from the carotid bodies, and removal of the pontine regions are key differences between in vitro and in vivo experiments. In that regard, activation of A_{2A} receptors at the carotid body level augments breathing in adult rats (12, 45, 48) (for a review, see Ref. 3). Blocking adenosine A_{2A} receptors produced a hypoventilation in young rats, an effect not observed in newborn or adult rats, which is consistent with our and other results showing that adenosinergic modulation of respiratory control changes over the course of

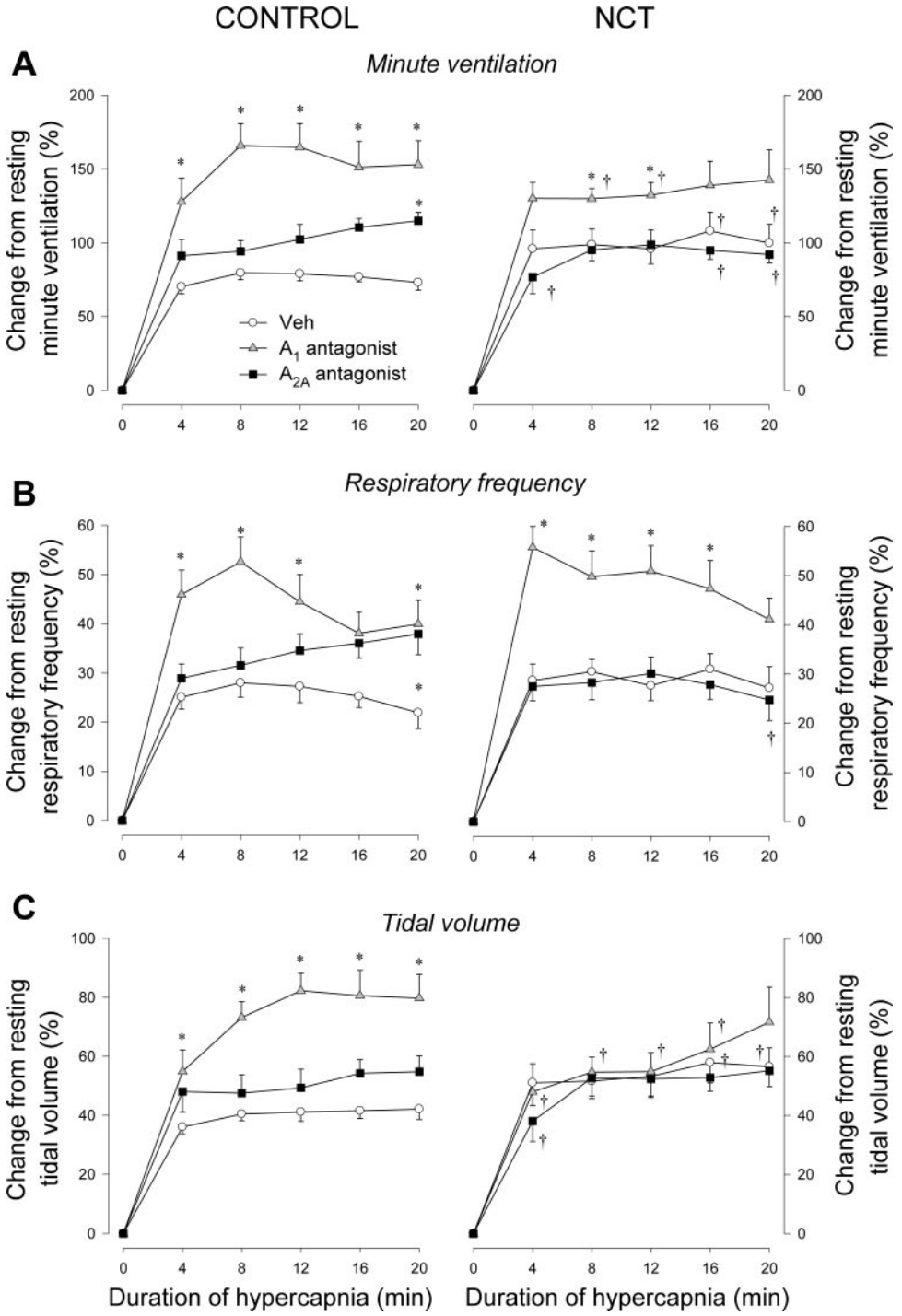

Fig. 4. Hypercapnic ventilatory responses after injection with either vehicle or A_1-receptor (DPCPX) or A_{2A}-receptor (ZM-241385) antagonists in control and NCT male young rats. The hypercapnic responses were expressed as %change from baseline in control (*left*) or NCT (*right*) rats. Values are means ± SE of vehicle (Veh; ○), A_1 antagonist (gray triangles), and A_{2A} antagonist (■). *A*: minute ventilation response. *B*: respiratory frequency response. *C*: tidal volume response. *Significantly different ($P < 0.05$) compared with respective vehicle value. †Significantly different ($P < 0.05$) compared with respective control rats.

development (44, 47). In brain stem-spinal cord preparations, removal of the pontine and other rostral structures, which contain a large quantity of adenosine A_{2A} receptors compared with other structures (61), may reduce the inhibitory effect of adenosine A_{2A} receptors and consequently reduce the excitatory effect of adenosine A_{2A} antagonist.

Hypercapnic ventilatory response. Ventilatory response to a moderate increase in CO_2 (5%) was enhanced by adenosine A_1-receptor inactivation. Although no previous studies have examined the role of specific adenosine-receptor subtypes in the hypercapnic ventilatory response of young rats, studies in adult humans demonstrated that systemic administration of a nonspecific adenosine-receptor antagonist (caffeine) increased the hypercapnic ventilatory response in adult humans (14, 54). The site where the adenosine antagonist caffeine exerts its acute effect in humans is unknown, and adenosine A_1 receptors are widely distributed in the central nervous system (58, 59). More precisely, adenosine A_1 agonist decreased c-Fos expression in the nucleus tractus solitarus, the area postrema, and the raphe obscurus and increased it in the parabrachial nucleus

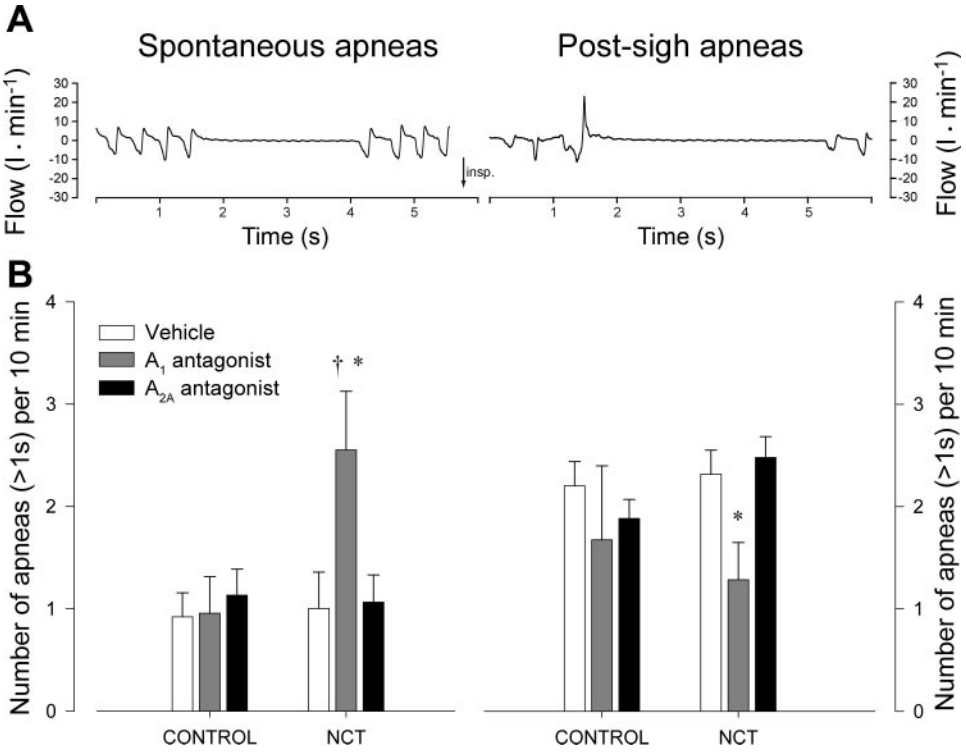

Fig. 5. Occurrence of spontaneous and postsigh apneas after injection of either vehicle or A_1-receptor (DPCPX) or A_{2A}-receptor (ZM-241385) antagonists in control and NCT male young rats. *A*: flow signal of a spontaneous apnea (*left*) and a postsigh apnea (*right*). *B*: mean values of number of apneas per 10 min ± SE of vehicle (open bars), A_1 antagonist (gray bars), and A_{2A} antagonist (closed bars). *Significantly different compared with respective vehicle value. †Significantly different compared with respective control rats. Values are significantly different when $P < 0.05$.

(62). These structures contain adenosine A_1 receptors (9, 23) and are putative central CO_2-chemosensitive sites (11, 18). Accordingly, we propose that inactivation of adenosine A_1 receptors prevents the inhibitory action of adenosine in specific CO_2-chemosensitive structures within the brain stem.

In contrast, inactivation of adenosine A_{2A} receptors had a small impact on the hypercapnic response. A_{2A}-receptor distribution is restricted to the striatum, the nucleus accumbens, and the olfactory tubercule (61). In the brain stem, these receptors were found in high quantity in the nucleus tractus solitarus and in medium quantity in the gigantocellular nucleus, the parapyramidal nuclei, the Bötzinger complex, and the locus ceruleus (61, 68). Because the nucleus tractus solitarus and the locus ceruleus are putative CO_2-chemosensitive sites (11), inactivation of A_{2A} receptors in these structures may explain the increase of the hypercapnic response observed in the present study. Because adenosine A_{2A} receptors are expressed only in few areas involved in CO_2 chemosensitivity, this receptor subtype might have a less important role in the hypercapnic ventilatory response than A_1 receptors. However, it cannot be ruled out that adenosine A_{2A} antagonist does not reach the brain in the same amount as A_1 antagonist, as discussed previously.

Impact of NCT on Adenosinergic Modulation of Respiratory Control

Ventilation at rest. Comparing mean data between control and NCT rats receiving vehicle injection yielded results similar to those reported previously by us (47): NCT had no effect on ventilation at rest. However, the use of breath density maps showed that NCT increased respiratory activity in young rats in a way identical to adults for a population of breaths (as observed in Fig. 2*A*, *right*), suggesting that, with maturation, young NCT rats will progressively achieve the same high respiratory activity as NCT adults (47). The functional consequences of this heterogeneous f_R observed in NCT rats are unknown, especially because this treatment does not affect the number of apneas. In humans, however, adenosine infusion produces periodic breathing during sleep (24). Consequently, treatments that interfere with adenosinergic neurotransmission may predispose to respiratory in-

Table 2. *Summary of effects of adenosine receptor inactivation on resting ventilation, apnea occurrence, hypercapnic ventilatory response, and $\dot{V}O_2$ in control and NCT rats*

	Control Rats					NCT Rats				
Adenosine Receptor Antagonist	Resting $\dot{V}_E$	SP Apneas	PS Apneas	CO_2 Response	$\dot{V}O_2$	Resting $\dot{V}_E$	SP Apneas	PS Apneas	CO_2 Response	$\dot{V}O_2$
A_1 (DPCPX)	—	—	—	↑ ↑	—	↑	↑ ↑ ↑	↓	↑	—
A_{2A} (ZM-241385)	↓	—	—	↑	—	—	—	—	—	—

SP, spontaneous apneas; PS, postsigh apneas; ↓ and ↑, Significant decrease (↓) or increase (↑) of >20%; ↓ ↓ and ↑ ↑, significant decrease (↓ ↓) or increase (↑ ↑) of >50%; ↑ ↑ ↑, significant change of > 100%; —, no change.

stability during sleep. This hypothesis requires further investigation.

Unlike controls, ventilation of NCT rats was increased by blocking adenosine A_1, but not A_{2A}, receptors (Table 2), suggesting that adenosinergic modulation of resting ventilatory activity (via A_1 receptors) is enhanced by NCT. Comparing control and NCT rats also showed that caffeine treatment abolished the depressant effect of the A_{2A}-receptor antagonist on resting ventilation, indicating that NCT influenced A_{2A}-receptor expression. This observation is consistent with results showing that chronic caffeine increases A_{2A}-receptor density in mouse brain (64). A_{2A} receptors are expressed in the nucleus tractus solitarus (61) and the carotid bodies (12), and recent results from our laboratory demonstrate that NCT increased adenosine A_{2A}-receptor mRNA in the carotid bodies of adult rats (Montandon G, unpublished observation). Consequently, because A_{2A} receptors are excitatory in the carotid bodies, an increase of their expression likely augments respiratory drive at rest and enhances $\dot{V}_E$. This interpretation likely explains why the A_{2A} antagonist dose used was not sufficient to decrease ventilation in NCT rats as observed in controls.

Hypercapnic ventilatory response. An important result of this study is that NCT decreased the impact of A_1 antagonist on the ventilatory response to hypercapnia (Table 2). Because $\dot{V}_{O_2}$ is not altered by caffeine, these changes are unlikely related to metabolism. In control rats, A_1 antagonist reduces the inhibitory effect of endogenous adenosine. In NCT rats, however, the same dose of adenosine A_1 antagonist was not sufficient to augment the hypercapnic response to the level observed in control rats. These results suggest that NCT increases adenosine A_1-receptor expression and/or enhances the capacity for endogenous adenosine release. NCT increased adenosine A_1-receptor expression in the brain stem of neonatal rats (23), and it has been proposed that, when caffeine occupies the adenosine receptor's binding site, the number of receptors increases to maintain efficiency of the adenosinergic system (7, 33). Such effects may underlie the influence of NCT on the A_1 antagonist group reported here. A small increase of adenosine A_{2A}-receptor expression in the nucleus tractus solitarus or in the locus ceruleus is likely because a decrease of V_T response to hypercapnia was observed in NCT rats. In fact, adenosine A_{2A} receptors are distributed in these brain stem structures (61), explaining the small impact of NCT on the response of rats injected with A_{2A} antagonist.

Occurrence of apnea. Adenosine plays an important role in apnea generation because adenosine agonist decreases spontaneous apneas in adult rats (49, 50). However, this effect may be sleep-wake state dependent because, as we mentioned previously, adenosine infusion during sleep produces periodic breathing. In the present study, however, adenosine antagonists did not change spontaneous apneas in control young rats. This can be explained by the low dose of antagonist used or by the young age of the rats. Furthermore, sleep-wake state may also be a factor, and this variable was not controlled in the present study. In NCT rats, however, A_1 antagonist increased the occurrence of spontaneous apneas. These data seem contradictory to the fact that caffeine is used to treat apneas in premature newborns. However, in this study, young rats are used instead of immature newborns. In fact, because developmental changes in neurotransmission occur around postnatal *day 12* in the respiratory brain stem nuclei of rats (67), the possibility that age-dependent effects of caffeine on ventilatory activity reflect maturational changes in neurotransmission is yet to be investigated.

The mechanism of action of neonatal caffeine on apneas is unclear. In humans, central apneas can be provoked by a decrease in arterial P_{CO_2} below apneic threshold (41). In human newborns, the eupneic threshold is very close to the CO_2 apneic threshold (35). High sensitivity to CO_2 after injection of A_1 antagonist could lead to hyperventilation and, consequently, to high variations of CO_2 levels. This may increase the risk to reach apneic threshold, especially in young animals, and thus enhance the occurrence of apneas. These results suggests that, after this treatment, absorption of a nonspecific antagonist such as caffeine may increase apneas in caffeine-treated young rats.

Adenosine A_1-receptor antagonist increased the occurrence of sigh in NCT rats. Despite this increase, adenosine A_1-receptor inactivation decreases the occurrence of postsigh apneas in NCT rats. This suggests that the mechanism that induces apneas after a sigh, rather than sigh occurrence, is altered by NCT. This mechanism is thought to be related to pulmonary stretch reflexes occurring after the sigh (46). Our results suggest that A_1, rather than A_{2A}, receptors are involved. Adenosine A_1 receptors are present in the nucleus tractus solitarus and the parabrachial nucleus (9, 23), two structures involved in pulmonary stretch reflexes (17). NCT increases adenosine A_1-receptor expression in the parabrachial nucleus of newborn rats, an effect not observed in young rats (23). In this study, however, NCT was administered during a shorter period of time (2–6 days old), which may explain the absence of effect of NCT. The cumulative effect of the likely NCT-induced overexpression of adenosine A_1 receptors in this structure and inactivation of adenosine A_1 receptors by a specific antagonist might explain the decrease of postsigh apneas observed in this study.

In conclusion, adenosine is an important neuromodulator of respiratory control. However, this study is the first to investigate the specific role of adenosine receptors in freely behaving young rats and shows that adenosine modulates both resting ventilation and CO_2 chemosensitivity. More specifically, data suggest that hypercapnia activates adenosinergic pathways, which attenuate responsiveness (and/or sensitivity) to CO_2 via A_1-receptor activation. We suggest that inactivation of A_{2A} receptors in the carotid bodies and/or nucleus tractus solitarius decreases the respiratory drive to ultimately reduce ventilation at rest. Furthermore, our study demonstrated that the adenosine A_1-receptor antagonist increases occurrence of spontaneous apneas in caffeine-treated young rats. Caffeine administration is a common treatment for respiratory instabilities in newborns, especially those born prematurely. Our data showing that NCT elicits developmental plasticity of adenosinergic modulation of respiratory activity raise questions about the potential consequences of subsequent caffeine absorption during childhood (via maternal milk) on respiratory activity (6). Such situation might increase vulnerability to respiratory disease associated with neural control dysfunctions such as sleep apnea or sudden infant death syndrome (30, 42).

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