IN MATURE ANIMALS, METHYLXANTHINES affect various processes of respiratory control. They enhance diaphragmatic contractility (40), central chemosensitivity, and also medullary blood flow, which is associated with changes in the medullary pH (for a review, see Refs. 1, 35, 44). In addition, methylxanthines also seem to act directly on the central respiratory network, resulting in an augmentation of ventilatory movements (2, 23, 27–29, 32).

The various stimulatory effects have led to the therapeutic use of methylxanthines in the treatment of insufficient respiratory activity. The most frequently used derivative of methylxanthine is aminophylline. Aminophylline acts at peripheral and central targets, but its central effects are dominant (12, 33). As a central respiratory stimulant, it is therefore often used to protect against the occurrence of various forms of central respiratory failures, such as postextubation apneas occurring after assisted mechanical ventilation (4), centrally caused sleep apnea (12), periodic breathing (14), or recurrent idiopathic apneas of infants (34). The latter form of apnea is fairly common in preterm infants and has been attributed to a delay in the maturation of central respiratory control mechanisms (24). Unfortunately, aminophylline is not always effective in all such preterm infants, and in ~50% of these cases it was ineffective (33). The reason for such variable efficacy remained unclear.

In the present study, we examined the hypothesis that the inconsistent therapeutic effects of aminophylline reflect changes in the developmental state of the central respiratory network. Such an assumption refers to our knowledge that the medullary respiratory network undergoes significant changes during early postnatal development, as seen in the pattern of respiratory activity (36), the response to hypoxia (3, 5, 19, 37), central chemosensitivity (22, 25), and sensitivity to various neuromodulators (16–18). To study the changes of the central respiratory network directly, we use a transverse brain stem slice preparation of mice of defined ages (16, 17, 36). This preparation contains the presumed respiratory rhythm-generating kernel network, the pre-Bötzinger complex (PBC) (42), and also the hypoglossal (XII) motor nucleus, which is rhythmic. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
cally activated through oligosynaptic connections in phase with respiratory activity (30, 36). Thus it is possible to analyze, under in vitro conditions, not only identified respiratory neurons but also the systemic respiratory motor output recorded from XII rootlets. Slices were obtained from postnatal day 1 until postnatal day 13 (P1–P13), a period that covers the most essential maturational stages. The respiratory network of mice is relatively immature at birth compared with humans (9) and matures quickly within 2–3 postnatal weeks. Therefore, the preparation can be used as a model for pre- and perinatal developmental changes of humans.

The data presented here demonstrate that aminophylline exerts differential modulatory effects on the frequency and amplitude of rhythmic respiratory and hypoglossal activity depending on developmental stage.

**METHODS**

**Preparation.** Female and male mice \( n = 14 \) of different postnatal ages (ranging from P1 to P13) were deeply anesthetized with ether, decapitated at the supracollicular level, and decorticated. The methods to prepare the transverse brain stem slices have been described elsewhere (36). Thus only the most important steps are summarized here. The brain stem was isolated in ice-cold artificial cerebrospinal fluid (aCSF) and secured on a vibratome, with its rostral end tilted downward at an angle of 20° to the plane of the razor blade. Thin slices (200 μm) were sectioned serially and discarded until the rostral boundary of the PBC (42) was reached, as recognized by typical cytoarchitectonic landmarks such as the inferior olive, the XII nucleus (Fig. 1A), and the facial nucleus. The following slice was cut 650–700 μm thick and immediately transferred into a recording chamber, submerged under a stream of aCSF containing (in mM) 128 NaCl, 3 KCl, 1.5 CaCl\(_2\), 1 MgSO\(_4\), 24 NaHCO\(_3\), 0.5 NaH\(_2\)PO\(_4\), and 30 n-glucose that was equilibrated with carbogen at 27°C to pH 7.4 (flow rate 10 ml/min). Because the preparation was isolated from afferent inputs, it was beneficial to increase the potassium concentration in the aCSF to 8 mM to increase neuronal excitability and to stabilize rhythmic respiratory activity lasting for several hours. The preparation was then allowed to stabilize for 30 min. Modulatory effects on respiratory activity were assessed by exchanging the superfusate to an aCSF containing aminophylline (Sigma) in a dosage of 20 μM.

Anoxia was induced over a period of 30 min by gassing the aCSF with 95% N\(_2\) and 5% CO\(_2\). After 30 min anoxia, gassing with 95% O\(_2\) restored O\(_2\) supply. Aminophylline was applied

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![Fig. 1. A: schematic illustration of the transverse brain stem slice from mice containing the pre-Bo\"tzinger complex (PBC), nucleus ambiguus (NA), nucleus tractus solitarius (NTS), inferior olive (IO), spinal trigeminal nucleus (Sp5), hypoglossal (XII) motor nucleus, and XII rootlet. The two arrows show the oligosynaptic projection from the PBC to the XII nucleus and its output in hypoglossal rootlets. B: XII rootlet activity was recorded extracellularly (middle) and integrated (top). In parallel, postsynaptic activity was recorded from an identified inspiratory neuron in PBC (bottom).](http://jab.physiology.org/)

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in a concentration of 20 μM to the aCSF 10 min before induction of anoxia.

Recording and data analysis. Systemic respiratory output activity was recorded extracellularly with a suction electrode from the central ends of XII rootlets and amplified (2000 times, Fig. 1B, middle), filtered (low pass 1.5 kHz, high pass 250 Hz), and electronically integrated (τ = 200–300 ms) (Fig. 1B, top). Neurons from the PBC (n = 14) were recorded in the whole cell voltage clamp configuration (Fig. 1B, bottom). Intrasonic recordings were distinguished in current clamp from intra-axonal recordings by the shape of action potentials and the presence of spontaneous postsynaptic activity. Patch electrodes manufactured from filamented borosilicate glass (Clarke GC 150 F) had a tip diameter of 1.5–2 μm and were filled with a solution containing (in mM) 140 D-gluconic acid (potassium salt), 1 CaCl₂, 10 EGTA, 2 MgCl₂, 4 Na₂ATP, and 10 HEPES (pH 7.3–7.4). Such electrodes had series resistances of 7–8 MΩ that were fully compensated. Negative current pulses (−1 nA, 50 ms) were applied at 1-s intervals to measure series resistance changes. The positive hydrostatic pressure was reduced when spikes were recorded extracellularly to control the approach to cell. Gigaseal formation was achieved by applying negative hydrostatic pressure to the electrodes. Respiratory neurons were usually recorded within a depth of 200–400 μm. Neurons were identified by their periodic burst discharges synchronized with respiratory XII nerve activity.

Current or voltage clamp measurements were performed with an Axopatch 200 amplifier (Axon Instruments). For analysis, we used only intracellular recordings with a membrane potential of less than −50 mV and small leak currents when neurons were voltage-clamped at physiological potentials of −60 to −70 mV. With strong ongoing respiratory activity, we sometimes observed escape of action potentials, which indicated that the space clamp conditions were not ideal. Synaptic currents, however, were not substantially affected, and therefore we accepted such situations to directly compare the amplitudes of respiratory synaptic drive currents at physiological voltage ranges.

Raw data were digitized by use of a DT 2821 interface (Scientific Solutions) and stored on videotape (VR-100,Instrutech) for off-line analysis. Drug-induced changes were measured and given as percentage changes from control values for each experiment. Frequency and amplitude of XII bursts were assessed from integrated rootlet activity by averaging 20 cycles before and during aminophylline application. Significance was determined by Student's t-test.

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**Fig. 2.** A: recording of rhythmic activity from the XII rootlet under control and after application of 20 μM aminophylline. B: aminophylline increased frequency without significantly changing the amplitudes of integrated XII bursts. C: aminophylline did not change the amplitudes of excitatory synaptic drive currents in neonatal animals (right) when compared with control conditions (left). The neuron was voltage-clamped at a membrane potential of −70 mV.
RESULTS

Bath application of 20 μM aminophylline increased the frequency of respiratory burst discharges of XII rootlets at an age of P1–P6. This is exemplified in a recording from a 2-day-old mouse in Fig. 2. The aminophylline effect reached maximum after 6–10 min, when the frequency of respiratory bursts was significantly elevated by 41 ± 25% (n = 6; P < 0.05). In contrast to this stimulatory effect on respiratory frequency, there were no obvious effects (2 ± 3%; n = 6) on the burst amplitude (see superimposed bursts in Fig. 2B).

Identical effects on respiratory burst frequency, but lack of effects on the amplitudes and durations of respiratory drive currents in PBC neurons as well as constancy of tonic interburst XII activity (see Fig. 2A), were taken as evidence that the aminophylline effects, as seen in hypoglossal discharges, originate from changes of respiratory network activity. Verification for this assumption is shown in Fig. 2C, which illustrates voltage clamp recordings from a neonatal (P2) PBC neuron at a holding potential of −70 mV. Aminophylline increased neither the amplitude, duration, nor temporal pattern of inspiratory synaptic drive currents compared with control conditions. Recordings from six neonatal inspiratory PBC neurons revealed a comparable response with maximally a small (10%) increase in the amplitude of excitatory synaptic drive currents.

In juvenile animals with an age of P7–P13, however, the same concentration of aminophylline (20 μM) evoked a significant increase (P<0.05) in the amplitude of respiratory XII bursts (31 ±18%) (n = 8). Respiratory bursts in XII rootlets increased in steepness and revealed a rapid peaking, and the duration of bursts was slightly prolonged (Fig. 3). In contrast to the effect in neonatal mice, there was, however, no significant effect on the frequency of respiratory XII bursts (6 ± 9%, n = 8; see Fig. 3A for a P9 slice). A comparable augmentation effect was seen on excitatory synaptic drive currents recorded in inspiratory PBC neurons. Aminophylline (20 μM) increased the amplitude of these drive currents by 66% (range 45–81%) and prolonged the duration of respiratory bursts by 34% (range 26–46%) (n = 3) (Fig. 3C). The temporal pattern of respiratory bursts remained relatively unchanged. These effects were fully reversible after washout of aminophylline (not shown). Similarity of responses of PBC respiratory interneurons and XII motoneurons and constancy or even reduction of tonic interburst XII activity (see Fig. 3A) again indicated a respiratory origin of the stimulatory effects.

Sequential histograms of the amplitude (Fig. 4, A and C) and frequency of integrated respiratory bursts in XII rootlets (Fig. 4, B and D) were performed to determine the time delay of aminophylline actions. This is shown for a neonatal (Fig. 4, A and B) and juvenile (Fig. 4, C and D) slice. The effects on burst
amplitudes in juvenile mice and on burst frequencies in neonatal animals reached significance ($P < 0.05$) 6–10 min after aminophylline application. The delays of the aminophylline effects in neonatal and juvenile animals were quite similar, indicating that there were no major differences in drug diffusion.

The time courses of maturational changes in aminophylline efficacy on frequency and amplitudes of respiratory XII bursts are illustrated in a plot of these functions against postnatal ages (Fig. 5). Each value in these graphs was obtained from averaging the amplitudes (Fig. 5A) and frequencies (Fig. 5B) of 20 bursts before and during the maximal effect of identical aminophylline concentrations. Note that the effect of aminophylline on the amplitude of hypoglossal respiratory bursts was evident only when animals were older than 7 days, whereas the effects on burst frequency seen in neonatal animals vanished at an age of 8–10 days.

**DISCUSSION**

Aminophylline, a drug most frequently used for the treatment of centrally caused apneic episodes, is often ineffective in prematurely born infants (8, 21, 24). In this presentation, we demonstrate on a functional mouse brain stem slice preparation that such variability reflects developmental changes of neurons in the respiratory center. We used a concentration of 20 μM aminophylline throughout the study, because this concentration corresponds with the therapeutic blood concentration range recommended in intensive care units for standard treatment of central apnea of preterm infants (2, 38). In early neonatal mice, we found that aminophylline fails to exert obvious effects on the amplitude but evokes a significant increase in the frequency of respiratory bursts. In animals that were older than P7, however, aminophylline induced an increase in the amplitude without significantly changing the frequency of respiratory bursts. Intracellular recordings from identified respiratory neurons of the PBC verified that such changes originate directly from the respiratory center and do not represent secondary or additional major effects on hypoglossal motoneurons. Therefore, an important question to be answered in future experiments concerns the processes that control respiratory frequency in premature animals, without changing the intensity of inspiratory burst activity.

The data are in general agreement with the description of comparable stimulatory effects of aminophylline or theophylline on respiration in vivo in adult rats, mice, and cats (10, 11, 25, 32). Such studies, performed on decerebrate, vagotomized, and paralyzed adult cats, have demonstrated a dose-dependent modulation of respiratory motor output in response to intravenous administration of aminophylline. Low doses of ami-
nophylline increased phrenic, hypoglossal, and recurrent laryngeal nerve activity, whereas high doses of aminophylline induced a marked increase only in phrenic nerve activity (6). In the en bloc brain stem-spinal cord preparations of rats (23), a comparable effect was seen in the theophylline-induced attenuation of hypoxic depression of respiratory frequency.

Although the experiments on more complex in vivo and in vitro preparations remain uncertain regarding the origin of such changes because of possible indirect effects through connected neural tissue, our experiments on the transverse brain stem slice preparation allow the effects to be referred directly to the isolated respiratory network. Our measures of constancy of tonic XII output activity and of identical responses in inspiratory burst activities in respiratory neurons and XII activity indicate the absence of major effects mediated indirectly through modulation of hypoglossal motoneurons. The only system that could be affected and cause indirect effects through its connections with the respiratory center is the raphe nucleus.

Although the present experiments reveal that aminophylline acts directly on the medullary respiratory center, the underlying mechanisms remain to be analyzed. There is good reason to assume that aminophylline is involved in the cAMP-mediated modulation of respiratory neurons (39, 41), and the classical view was that the molecular processes involved blockade of phosphodiesterase, which led to elevation of intracellular cAMP levels (26, 31). Now, however, we know that xanthines do not act on intracellular nucleotidases and phosphatases at the low micromolar concentration used in the present experiments. Following the description of Fredholm et al. (15), we have to assume that aminophylline binds to adenosine A1 receptors to block their activation. Such binding to pre- and postsynaptic A1 receptors, which are persistently activated in the respiratory network (15, 41), will inhibit adenylyl cyclase to release K^+ channels from activation and to induce disinhibition of voltage-regulated Ca^{2+} channels. Thus respiratory network activity will be augmented. The finding that such effects are not seen in neonatal ages points to late expression of adenosine receptor and/or immaturity of their signal pathway in early neonatal animals.

Aminophylline enhances respiratory motor output activity across a wide range of animal species, such as mature rats, cats, and mice. Such identical findings in different animal models may qualitatively predict similar respiratory effects in humans. Indeed, measurements of the excursions of the diaphragm in human neonates have indicated that aminophylline increases diaphragmatic excursions by 43% without changing the respiratory rate (20). Therefore, the increase in the amplitude of the respiratory motor output, as described in this study of juvenile preparations, is consistent with the clinical finding that aminophylline is effective in the treatment of neonatal humans who suffer from weak respiratory drive. In such neonatal patients, aminophylline treatment induces deepening of inspiratory movements, which leads to improved lung ventilation.

In neonatal mice, however, we observed an increase in respiratory frequency rather than in respiratory amplitudes. Because mice are born with a fairly immature respiratory network compared with the human respiratory network (9), it is an interesting assumption that certain preterm infants with a premature respiratory network may also respond to aminophylline with only an increase in respiratory frequency but not with deepening of breaths. This might be a harmful effect, because an increase in respiratory frequency in superficially breathing preterm infants may induce a fall of arterial partial pressure of CO_2 rather than a change in arterial partial pressure of O_2, which may even reduce breathing as a result of diminished chemoreceptive drive (13). Cordoba and co-workers (7) have shown that aminophylline treatment in preterm infants indeed leads to a reduction of the end-tidal partial pressure of CO_2 from 44 ± 7 to 38 ± 6 mmHg.

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**Fig. 5.** Maturational changes of the effects of 20 μM aminophylline illustrated as percentage change of amplitudes (A) and frequencies (B) of hypoglossal bursts. Each value represents the mean activity of 20 cycles as measured during control or during the peak effect of aminophylline. Note that effects on burst amplitudes were only seen in mature animals whereas changes in burst frequencies were significant only during neonatal ages.
The similarity between the respiratory responses of neonatal mice and preterm human infants suggests that this animal model will be very useful for studying the maturation of modulatory pathways in individual respiratory neurons and of the systems involved in frequency and amplitude control of breathing and also for developing novel drugs that may be more beneficial for the treatment of idiopathic apnea in preterm human infants.

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