Species differences in respiratory rhythm generation in rodents

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Gajda BM, FongAY, MilsomWK. Species differences in respiratory rhythm generation in rodents. Am J Physiol Regul Integr Comp Physiol 298: R887-R898, 2010. First published January 13, 2010; doi:10.1152/ajpregu.00339.2009.—We examined the role of riluzole (RIL) and flufenamic acid (FFA)-sensitive mechanisms in respiratory rhythmogenesis in rats and hamsters using the in situ artificially perfused preparation. Based on the hypothesis that respiratory networks in animals capable of autoresuscitation would have a greater prevalence of membrane mechanisms that promote endogenous bursting, we predicted that older (weaned) hamsters (a hibernating species) would behave more like hamsters. Consistent with this, we found that respiratory motor output in weaned hamsters [postnatal (P) 21] was highly sensitive to RIL (0.2–20 μM), while in young rats (P12–14) it was less so (only affected at higher concentrations of RIL), and weaned rats were not affected at all. On the other hand, respiratory motor output was equally reduced by FFA (0.25–25 μM) in both young and weaned rats but was unaffected in weaned hamsters. Coapplication of RIL and FFA (RIL + FFA) produced greater inhibition of respiration in both young and weaned rats compared with either drug alone. In contrast, in weaned hamsters, FFA coapplication offset the inhibitory effect of RIL alone. Increasing respiratory drive with hypercapnia/acidosis ameliorated the respiratory inhibition produced by RIL + FFA in weaned rats but had no effect in young rats.

Data from the present study indicate that respiratory rhythmogenesis in young rats is more dependent on excitatory RIL-sensitive and FFA-sensitive mechanisms than older rats and that fundamental differences exist in the respiratory rhythmic mechanisms between rats and hamsters.

Many species lose the ability to autoresuscitate from hypothermic respiratory arrest. To achieve this aim, we examined the effect of RIL and FFA on respiratory rhythmogenesis in three groups: 1) young rats [postnatal (P) 12–14], 2) weaned rats (P24–30), and 3) weaned hamsters (P24–30). The age groups of the rats were chosen to be on either side of the developmental transition (P16–18; Ref. 1) when the ability to autoresuscitate from hypothermic respiratory arrest is lost. Weaned hamsters, age matched to weaned rats, retain the ability to autoresuscitate from hypothemic respiratory arrest. These experimental groups allowed us to examine the relative role of RIL- and FFA-sensitive mechanisms in respiratory rhythm generation in rodents.
S. M. Paton, T. B. Spratt, and J. A. Stein

Respiratory Rhythmogenesis in Rats and Hamsters

METHODS

All experimental protocols were approved by the Animal Care Committee of the University of British Columbia under the guidelines set by the Canadian Council for Animal Care.

In Situ Arterially Perfused Working Heart-Brainstem Preparation

Experiments were performed on male Sprague-Dawley rats (Rattus norvegicus; University of British Columbia Rodent Breeding Facilities) or Syrian hamsters (Mesocricetus auratus; Charles River, Wilmington, MA), using the in situ arterially perfused working heart-brainstem preparation (34). We used three groups of animals: (1) young rats (P12–14; 20–41 g), (2) young rats (P24–30; 68–107 g), and (3) weaned hamsters (P24–30; 33–100 g). In brief, each animal was deeply anesthetized by halothane or isofluorane via spontaneous inhalation. The depth of anesthesia was gauged by lack of limb withdrawal to noxious pinch. The animal was then transected subdiaphragmatically, and the anterior portion of the body was placed in chilled modified Ringer solution bubbled with carbogen (95% O2-5% CO2) gas, followed by decerebration at the precollicular level and the skin removed. The left phrenic nerve (PHR) was isolated and cut at the level of the diaphragm. In a number of preparations, the right vagus nerve (CVN) was also isolated for recordings to establish the viability and stability of these preparations. The preparation was then transferred to an acrylic recording chamber, and the descending aorta was cannulated with a double lumen catheter (Brain-tree Scientific, Braintree, MA). Perfusion began immediately with a modified Ringer solution bubbled with carbogen gas (95%O2-5% CO2) and warmed to 31–32°C by an inline heat exchanger. Neuromuscular blockade was achieved using either rocuronium bromide (2 mg Zemuron) or vecuronium bromide (0.6 mg Norcuron) added directly to the perfusate.

Whole nerve activity from the PHR and the CVN (when dissected) was recorded via glass suction electrodes. Raw neurograms were passed through preamplifiers (FRAMP PRA-1, -2) and then amplified (total amplification = ×2,000) and filtered (500 Hz-1 kHz, FRAMP GPA-1). Perfusion pressure was measured via one lumen of the catheter attached to a physiological pressure transducer (Narco Scientific) connected to an amplifier (Gould Universal). Amplified nerve signals and perfusion pressure were sampled at 2 kHz and recorded by Windaq data acquisition software (DataQ Instruments, Akron, OH). Only preparations showing a ramping PHR discharge were included in this study.

Experimental Protocol

Effect of RIL and FFA on respiration in the in situ arterially perfused preparations. All preparation groups were exposed to the same protocol. After the perfusion started, the preparations were allowed to stabilize for 30–60 min before any drug administration. Three series of protocols were performed; one on each of a subset from each preparation group. The drugs RIL (0.2–20 μM) and FFA (0.25–25 μM) were applied in progressively increasing concentrations to the perfusate individually [RIL alone or FFA alone (subsets 1 and 2)] and in combination [RIL + FFA (subset 3)]. The concentrations of the drugs used and the incremental changes in concentration are listed in Table 1. Following each stepwise increase in concentration of the drugs, the preparation was allowed to stabilize for ~5–15 min before addition of the next dose. Only one drug treatment protocol was applied to each preparation. In all preparations, the lowest concentra-

Table 1. Concentration increments of drugs used in the in situ preparations

<table>
<thead>
<tr>
<th>RIL Alone</th>
<th>FFA Alone</th>
<th>RIL + FFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.25</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>1.25</td>
<td>7.5</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>10</td>
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<td>6</td>
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<tr>
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<td>12.5</td>
<td>20</td>
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<tr>
<td>12</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>14</td>
<td>17.5</td>
<td>20</td>
</tr>
<tr>
<td>16</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>18</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

RIL, riluzole; FFA, flufenamic acid.

FFA-sensitive mechanisms on respiratory rhythm generation over development within the same species (young rats vs. weaned rats), as well as between two species at similar levels of development (weaned rats vs. weaned hamsters; Refs. 4, 5), yet with different ability for autoresuscitation from hypothermic respiratory arrest.

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tion of drug was added to the perfusate and the concentration was increased in stepwise increments (as listed in Table 1) until either 1) the end of the drug concentration range, or 2) respiratory motor output could not be detected for at least 5 min. The number of preparations within each preparation group in each drug treatment is stated in RESULTS. Time and vehicle controls (n/H11005/6 for each vehicle), where the equivalent amount of solvent used for each drug, both individually and in combination, was added to the perfusate using the same experimental timeline as in the drug protocol, were also performed.

**Effect of increasing respiratory drive.** In another series of experiments, we determined whether increased respiratory drive could ameliorate the effect of RIL/FFA applied in combination. The preparations were allowed to first stabilize for 30–60 min under normocapnic conditions (“normal drive,” as described above) before respiratory drive was increased by bubbling the perfusate with 7% CO2–93% O2 (“high drive”) resulting in an increase in the PCO2 of the perfusate from ~40 to ~52 Torr. This also resulted in a concomitant decrease in pH from 7.4 to approximately pH 7.2. The preparations were allowed to stabilize in this hypercapnic/acidotic environment for another 20–30 min at high drive before the drugs RIL + FFA were added in combination using the same protocol described above.

**Drugs and Solutions**

RIL (2-amino-6-trifluoromethoxy benzothiazole; Sigma, St. Louis, MO) was solubilized in 1.0 M hydrochloric acid and heated while stirring until the drug dissolved. The solution was diluted to a concentration of 2 mM with distilled, deionized water. FFA (Sigma) was solubilized with 100 mM sodium hydroxide and titrated to pH 7.4–8.0 with 100 mM HCl. This solution was then diluted to 5 mM with distilled, deionized water. The Ringer solution, containing 125 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 2.5 mM CaCl2, 1.25 mM MgSO4, 1.25 mM KH2PO4, and 1 mM D-glucose, was made fresh each day. A high molecular weight compound (1.25%, Ficoll, type 70, 70 kDa; Sigma) was added to the solution as an oncotic agent.

**Data and Statistical Analysis**

All data were recorded online using commercially available data acquisition hardware and software (DI200 analog-to-digital converter, Windaq software; DataQ). Analysis was performed offline using Spike2 software (v 4.24; Cambridge Electronic Designs, Cambridge, UK). The CVN discharge was not quantified and was used only for validation (Fig. 1). Raw PHR nerve recordings were full-wave rectified and smoothed. Analysis of the smoothed PHR neurograms mea-

Table 2. Baseline phrenic nerve burst discharge of weaned rat, young rat, and weaned hamster in situ preparations

<table>
<thead>
<tr>
<th>Preparation Type</th>
<th>n</th>
<th>Burst Frequency, bursts/min</th>
<th>T1, s</th>
<th>TE, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weaned rat</td>
<td>53</td>
<td>18.6 ± 1.0</td>
<td>0.81 ± 0.04</td>
<td>3.04 ± 0.3</td>
</tr>
<tr>
<td>Young rat</td>
<td>77</td>
<td>19.4 ± 1.0</td>
<td>0.51 ± 0.02†</td>
<td>3.25 ± 0.2</td>
</tr>
<tr>
<td>Weaned hamster</td>
<td>42</td>
<td>45.2 ± 4.0††</td>
<td>0.51 ± 0.04‡</td>
<td>1.28 ± 0.1‡‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = number of preparations. Weaned rats, >21 postnatal days; young rats, 12–14 postnatal days; weaned hamsters, >21 postnatal days; T1, inspiratory duration; TE, interburst interval. All statistical analyses were performed using a one-way ANOVA on ranks, followed by a Dunn’s post hoc test. *P > 0.05, weaned rats vs. young rats. †P > 0.05, weaned hamsters vs. young rats. ‡P > 0.05, weaned hamsters vs. weaned rats.

Fig. 2. Effect of increasing concentration of riluzole (RIL) on respiratory output in the in situ preparation. Representative continuous traces of rectified and smoothed phrenic neurograms (JPFR) and raw PHR neurograms from a weaned rat [postnatal (P) 25; A], a young rat (P12; B), and a weaned hamster (P26; C) in response to increasing concentrations of RIL are shown. A’–C’; representative rectified and smoothed PHR neurograms at different concentrations of RIL. Traces are averaged cycle waveforms of ~10 bursts, calculated from the onset of phrenic nerve discharge.
sure: \( T_i \), inspiratory duration in seconds defined as the time between the onset and end of each phrenic burst; \( T_e \), interburst interval (expiratory duration) in seconds defined as the time between the end of one phrenic burst and the onset of the following burst; and burst amplitude, in arbitrary units defined as the height of the peak of the burst in the rectified smoothed neurograms. Phrenic burst frequency was calculated from the sum of \( T_i \) and \( T_e \) (bursts/min). Fictive neural ventilation (n\( \dot{V} \dot{E} \)) was calculated by multiplying PHR burst amplitude and (expiratory duration) in seconds defined as the time between the end of one phrenic burst and the onset of the following burst; and burst amplitude, in arbitrary units defined as the height of the peak of the burst in the rectified smoothed neurograms.

**RESULTS**

Figure 1 shows representative traces of simultaneous recording of PHR and CVN discharge from weaned rat, young rat, and weaned hamster preparations. All three preparations were stable and viable as indicated by a ramping PHR discharge pattern and a three-phase respiratory rhythm (inspiration, postinspiration, and late expiration) as characterized by the discharge pattern in the CVN (34). The postinspiratory phase is characterized by a burst of neural activity in the CVN at the end of inspiration that decreases abruptly, while late expiration is defined as the period of quiescence in the CVN neural discharge (Fig. 1).

The baseline values for variables describing phrenic burst activity of the three groups of preparations are shown in Table 2. Weaned hamsters had a significantly higher baseline phrenic burst frequency than both young rats and weaned rats due to significantly shorter interburst intervals (\( T_e \)). No differences existed between the baseline frequencies of young rats and weaned rats. Weaned hamsters and young rats also had shorter burst durations than weaned rats.

**Effect of RIL**

Figure 2 shows representative traces of continuous PHR recordings from the three preparations with increasing concentrations of RIL. In weaned rats (\( n = 6 \)), RIL had no effect on phrenic burst frequency (Figs. 2A and 3A) but did eventually cause a decrease in burst amplitude (Figs. 2A and 3B) and fictive n\( \dot{V} \dot{E} \) at the highest concentrations of RIL used (\( \geq 18 \mu M \); Fig. 3C). Increasing RIL had no effect on the burst shape of the PHR discharge until 20 \( \mu M \) RIL when the burst shape changed from ramping to rounded (Fig. 2A').

In young rats (\( n = 11 \)), concentrations of RIL (\( \geq 10 \mu M \)) decreased phrenic burst frequency (Figs. 2B and 3) primarily due to increases in \( T_e \), with little change in \( T_i \) (Fig. 2B). Burst amplitude also declined significantly at \( \geq 12 \mu M \) RIL (Fig. 3B) and n\( \dot{V} \dot{E} \) was significantly lower than baseline at 8 \( \mu M \) RIL. Only 5 of 11 (45%) preparations maintained respiratory motor output at \( \geq 12 \mu M \) RIL, while only 3 of 11 (27%) preparations
still produced rhythmic respiratory activity at 20 μM RIL. Rhythmic phrenic activity was lost due to a reduction in both phrenic burst frequency and amplitude. Weaned hamsters (n = 6) were highly sensitive to RIL with a 25% reduction in phrenic burst frequency at 0.2 μM RIL (Figs. 2C and 3A) due to an increase in T_E with no change in T_I (Fig. 2C). Burst amplitude also decreased from baseline at ≥5 μM RIL (Figs. 2C’ and 3B) and nV˙E was almost halved at 2 μM RIL (Fig. 3C). Only three of six (50%) weaned hamster preparations continued to produce respiratory motor output at 8 μM RIL, and by 14 μM RIL all preparations ceased rhythmic respiratory activity.

Comparing the response of the three preparation groups to RIL, weaned hamsters were the most sensitive to RIL followed by the young rats, while the weaned rats were barely affected by RIL (Fig. 3).

Effect of FFA

Figure 4 shows representative continuous PHR recordings from the three groups of preparations at increasing concentrations of FFA. In weaned rats (n = 7), increasing FFA up to 25 μM had no effect on burst frequency or burst shape (Fig. 5). However, FFA significantly reduced burst amplitude (≥0.2 μM) and nV˙E (≥5 μM; Figs. 4A and 5).

In young rats (n = 10), increasing concentrations of FFA had no effect on phrenic burst frequency (Figs. 4B and 5A), while concentrations ≥10 μM FFA decreased burst amplitude and nV˙E (Figs. 4B and 5). At 25 μM FFA, however, phrenic burst amplitude in 3 out of 10 young rat preparations declined and could not be measured above the background noise.

In weaned hamsters (n = 7), increasing the concentration of FFA had no effect on phrenic burst frequency. Burst amplitude and nV˙E were reduced slightly from the baseline at ≥15 μM FFA (Figs. 4C and 5).

Comparing the response of the three preparation types to FFA, the burst amplitude of weaned rats was the most sensitive, followed by young rats, although this was not statistically significant. Weaned hamsters were affected minimally by FFA.

Effect of Coapplication of RIL and FFA

Normocapnia: 5% CO₂ (normal drive). A range of concentrations of RIL and FFA was chosen to examine the effects of combined application of the drugs. Representative continuous PHR recordings from the three groups of preparations in response to coapplication of RIL and FFA (RIL + FFA) are shown in Fig. 6. In weaned rats (n = 10), RIL + FFA decreased phrenic burst frequency, amplitude, and hence nV˙E (Figs. 6A and 7). The reduction in phrenic burst frequency was mediated predominantly by an increase in T_E, with little effect on T_I (Fig. 6A’). The PHR burst retained a ramping discharge shape until the highest concentrations of RIL + FFA (Fig. 6A’). In 7 of 10 (70%) preparations from weaned rats, application of 12 μM RIL + 10 μM FFA abolished respiratory motor output by a progressive reduction in burst amplitude.

Fig. 4. Effect of increasing concentration of flufenamic acid (FFA) on respiratory output in the in situ preparation. Representative continuous traces of rectified and smoothed PHR and raw PHR neurograms from a weaned rat (P25; A), a young rat (P14; B), and a weaned hamster (P29; C) in response to increasing concentrations of FFA are shown. A’–C’: representative rectified and smoothed PHR neurograms at different concentrations of FFA. Traces are averaged cycle waveforms of ≥10 bursts, calculated from the onset of phrenic nerve discharge.
until it could no longer be detected above the baseline neural signal on the PHR.

In young rats \((n = 16)\), RIL + FFA also significantly reduced phrenic burst frequency by increasing \(T_\text{E}\). Phrenic burst amplitude and \(nV\dot{E}\) were also significantly reduced at all drug concentrations (Figs. 6B and 7). The PHR discharge shape was not affected by RIL + FFA until concentrations of \(>10\) \(\mu M\) RIL + 8 \(\mu M\) FFA (Fig. 6B’). Coapplication of RIL + FFA stopped respiratory motor output in 9 of 16 (56%) preparations at 10 \(\mu M\) RIL + 8 \(\mu M\) FFA, and at 20 \(\mu M\) RIL + 15 \(\mu M\) FFA in 100% of the preparations burst amplitude decreased until the bursts could not be detected above the baseline signal of the PHR.

In weaned hamsters \((n = 8)\), RIL + FFA also decreased phrenic burst frequency, amplitude, and \(nV\dot{E}\) (Figs. 6C and 7). The reduction in burst frequency was due to increases in \(T_\text{E}\) as \(T_1\) remained unchanged (Fig. 6C’). In four of eight (50%) weaned hamster preparations, respiratory motor output could not be detected at 20 \(\mu M\) RIL + 15 \(\mu M\) FFA due to a decline in frequency, while burst amplitude was maintained although reduced from baseline (Fig. 7).

Hypercapnia/acidosis: 7% \(CO_2\) (high drive). When we increased respiratory drive by bubbling the perfusate with 7% \(CO_2\), to mimic respiratory acidosis, burst frequency increased in both young rat and weaned rat preparations primarily due to a reduction in interburst interval \((T_\text{E})\). The same level of acidosis had no effect in weaned hamsters (Table 3).

In weaned rats, increased respiratory drive ameliorated the effect of the drugs on both phrenic burst frequency and amplitude and, consequently, \(nV\dot{E}\) (Fig. 8). With high drive, 100% of preparations continued to produce rhythmic respiratory activity under the highest concentration of the drug cocktail, compared with only 30% with normal drive.

In young rats, increasing respiratory drive did not alter the effect of RIL + FFA on phrenic burst frequency, amplitude, or \(nV\dot{E}\) (Fig. 8). Increasing respiratory drive did maintain respiratory activity in 15% of young rats at the highest concentration of the drug cocktail where all preparations had ceased respiratory bursting under normal drive.

In weaned hamsters, increasing respiratory drive had no effect on phrenic burst frequency, burst amplitude, or \(nV\dot{E}\) (Fig. 8). Increasing respiratory drive also did not increase the viability of weaned hamster preparations; 63% (5/8) of preparation produced rhythmic respiratory bursts at the highest concentration of the drug cocktail, compared with 50% (4/8) of preparations under normal drive (Fig. 8).

**DISCUSSION**

The present study examined the role of RIL- and FFA-sensitive mechanisms on respiratory rhythm generation in animals with different abilities to autoresuscitate from hypothermic respiratory arrest. We have a number of significant findings: 1) respiratory rhythm generation in hamsters was more reliant on RIL-sensitive processes than in rats; 2) respiratory rhythmogenesis in young rats was more reliant on RIL-sensitive processes than in weaned rats; 3) respiratory burst amplitude was modulated by FFA-sensitive mechanisms in rats but not in hamsters; 4) coapplication of RIL and FFA had an additive effect in rats, but in contrast, FFA offset the depressive effects of RIL in hamsters; and 5) increased respiratory drive ameliorated the effects of RIL and FFA on respiratory rhythm only in weaned rats but not in young rats or weaned hamsters. These data demonstrate changes in the cellular and synaptic mechanisms involved in the generation of respiratory rhythm over development in rats and between rats and hamsters that will be discussed below.

**Technical Considerations**

RIL has been used extensively to study the role of \(I_{\text{NaP}}\) in respiratory rhythmogenesis in rats and mice. However, RIL is not pharmacologically selective and affects a host of other currents/channels, including the transient sodium current \(I_{\text{NaT}}\).
background potassium channels (TREK-1 and TRAAK), and glutamatergic neurotransmission (2, 3, 10, 11, 25, 39, 49). At concentrations of RIL sufficient to block all $I_{NaP}$ (Refs. 39, 48), RIL will also have nonspecific effects throughout the respiratory network. At low concentrations, however, RIL has greater selectivity for $I_{NaP}$ (9–11, 39, 48, 49) and has little to no effect on glutamate receptor evoked currents (7, 10). Furthermore, although it is not possible to distinguish the effects of RIL on $I_{NaP}$ and $I_{NaT}$ (the same channel produces both $I_{NaP}$ and $I_{NaT}$; Ref 39), the effect of inhibiting $I_{NaT}$ on respiratory rhythm would be expected to be minimal, as there is an apparent large excess of $I_{NaT}$ for action potential generation (39). Therefore, at the concentrations used in this study (0.5–20 μM), RIL will have definitely blocked $I_{NaP}$ in rats, but the effects observed at concentrations ≥10 μM are likely due, at least in part, to blockade of other currents and glutamatergic neurotransmission (7, 10).

The drug FFA has been widely used for the study of $I_{CaN}$ currents in respiratory rhythm generation in rats and mice despite a range of nonspecific effects. Concentrations of FFA that completely abolish $I_{CaN}$ (≥100 μM; Ref. 17, 32) also block gap junctions (EC$_{50}$ = 40–47 μM) (20, 44) and affect multiple currents including large conductance Ca$^{2+}$-activated K$^+$ channels (16, 22), which can influence neuronal firing. Thus the concentrations of FFA used in the present study (≥25 μM) would have been insufficient to completely block $I_{CaN}$ and could have affected a number of other currents and channels besides $I_{CaN}$.

There is a dearth of knowledge of the effects of RIL and FFA on neuronal cellular mechanisms in other rodent species, including hamsters. The majority of the current literature on the effect of RIL and FFA on ionic currents has been derived from studies on mice and rats. However, an earlier study found that RIL had similar effects on neurotransmitter release in mouse, rat, and human neocortex, suggesting that RIL has similar effects across a wide range of species (21). Thus, while it is quite likely that RIL is acting via similar mechanisms in hamsters, direct studies on the effect of RIL and FFA on hamster respiratory neurons are required to substantiate this.

**Developmental Changes in Respiratory Rhythmogenic Mechanisms in Rats**

One of the primary aims of the current study was to examine if changes occur in the role of RIL- and FFA-sensitive mechanisms in respiratory rhythm generation over postnatal development. Any such change could contribute to the developmental loss of the ability to autoresuscitate from hypothermic respiratory arrest. We explored this transition using rats on either side of the developmental window when the ability to autoresuscitate is lost (1).

We hypothesized that the respiratory network of animals that can autoresuscitate are more excitable due to increased levels of basal drive from RIL- and/or FFA-sensitive mechanisms (9). Thus we predicted that young rats, which are capable of autoresuscitation from hypothermic respiratory arrest (1),
...would have greater reliance on RIL- and FFA-sensitive mechanisms for respiratory rhythm generation.

Consistent with the current literature, in the present study FFA-sensitive mechanisms were involved in the production of motor output (burst amplitude) but were not involved in respiratory rhythogenesis in either ages of rats (32, 33, 45). The effect of FFA on burst amplitude could have been due to a reduction in drive potential at the preBöTc (32) or reduced excitability of neurons resulting in reduced network activity (32, 42) leading to a decreased motor output. Similarly, the reduction in burst amplitude by RIL could have been due to reductions in intraburst spike production or a reduction in excitatory neurotransmission in respiratory rhythmic regions or respiratory premotor and motor neurons in the medulla or spinal cord (33, 39).

RIL-sensitive mechanisms were not required for respiratory rhythogenesis in weaned rats, consistent with previous studies using the in situ preparation (35, 43, 46), neonatal transverse brainstem slice preparations (8, 9, 37), and neonatal rat brainstem-spinal cord preparations (14). In contrast, RIL significantly reduced burst frequency in young rats, abolishing fictive respiratory activity in >70% of preparations. The effects on respiratory output in young rats were prominent at concentrations of RIL >10 μM, suggesting this was likely due to the effects of RIL on excitatory neurotransmission through mechanisms other than sodium currents (see previous section) such as inhibition of glutamate release (7, 10, 21). They could also be due to the action of RIL at a variety of sites including cessation of respiratory rhythm in rhythmicogenic sites (e.g., preBöTc); a reduction in drive from upstream regulatory sites (e.g., midline raphé; Refs. 33, 38); a failure in the transmission of the signal downstream in the respiratory network, including premotor neurons (39); or a combination of these effects. Systemic application of RIL precludes identification of the exact site of action without direct neuronal recordings within regions such as the preBöTc.

The depression of respiratory rhythm by RIL in young rat preparations in our study is in contrast with reports in the current literature from neonatal transverse brainstem slice preparations (8, 9, 37), neonatal rat brainstem-spinal cord preparations (14), or older in situ preparations (35, 43, 46). A recent study (43) found RIL reduced phrenic burst frequency of in situ preparations with the pons removed and may suggest that the pons in our young rat preparations were nonfunctional, possibly due to inadequate perfusion. However, our young rat preparations exhibited ramping phrenic discharge (Figs. 1 and 2) and possessed a three-phase rhythm (Fig. 1) indicating adequate perfusion of the in situ preparation (34). In addition, time and vehicle controls maintained phrenic bursting throughout the equivalent experimental duration, further supporting the viability of our young rat preparations.

In comparison with the transverse slice and the brainstem-spinal cord preparations, the in situ preparation has a more intact respiratory network. The reduction in respiratory rhythm in young rat preparations could be due to the effects of RIL on regions that provide tonic drive to the respiratory network, such as the retrotrapezoid nucleus (18) or pontine regions, which are absent in more reduced preparations. In addition, the age of the preparations in the present study was older than the neonatal slice or brainstem-spinal cord preparations (P12–14 vs. P5); thus the effect of RIL in our young rat preparations could reflect developmental increases in the expression of RIL-sensitive mechanisms at respiratory rhythmicogenic sites, such as the preBöTc (52), or at regions that provide drive to the respiratory network (9, 12, 19, 33, 38).

Previous studies (26, 27, 41, 52) provide abundant evidence indicating a developmental transition occurs at the end of the second postnatal week, the age of our young rat preparations. Associated with this transition are profound changes in the...
physiological responses to respiratory challenges (26, 41) and changes in the expression of a number of neurochemical systems, including the glutamatergic, GABAergic, and serotonergic systems in respiratory nuclei such as the preBotC (52).

In particular, a transient shift in the balance between excitatory and inhibitory neurotransmission at this age (52) could result in a reduction in tonic glutamatergic drive in the respiratory network. This imbalance could make rats at this age particularly sensitive to antagonism or interruptions of glutamatergic neurotransmission by RIL and is consistent with the increased sensitivity of our young rat preparations to RIL compared with other ages.

Coapplication of RIL and FFA resulted in similar decreases in respiratory rhythm and phrenic burst amplitude in both young and weaned rats, consistent with previous studies (9, 33, 36). At high concentrations, the drug cocktail abolished respiratory output in young rats, while weaned rats were able to maintain fictive respiration. Interestingly, in both groups of rats, the reduction in nV: following RIL + FFA administration was greater than what would be expected if the effect of the drug cocktail was merely an addition of the individual effects. This increased effect of drug combination was particularly notable in weaned rats where application of either RIL or FFA had little effect on phrenic frequency, while the application of both RIL and FFA drugs together resulted in significant reductions in phrenic burst frequency (Fig. 7). Therefore, the respiratory network in weaned rats appears to be able to compensate when one mechanism was removed but was severely compromised when both RIL-sensitive and FFA-sensitive mechanisms were blocked.

Earlier studies (9, 33) in neonatal mouse transverse slice preparations found that respiratory arrest following RIL + FFA administration could be restored by increasing respiratory drive, suggesting that the effect of the drugs was due to a reduction in drive. We wondered whether the same might be true in the present study. Increasing drive by hypercapnia/acidosis promoted basal respiratory activity in weaned rats and ameliorated the effect of the drugs, consistent with earlier studies (9, 33). This recovery of respiratory activity supports the notion that the depressing effects of RIL- and FFA-sensitive mechanisms on respiratory neurons could be overcome by increasing drive by activation of central chemoreceptors (18, 19, 30, 33, 38) and/or peripheral chemoreceptors (6, 13, 24). In contrast, while hypercapnic/acidotic stimulation increased the basal respiratory rhythm of young rats, this increase in respiratory drive was insufficient to overcome the effect of RIL + FFA. The failure of increasing drive to restore respiratory activity in young rat preparations may be a reflection of the weaker hypercapnic ventilatory response in rats of this age (41).

A second aim of this study was to determine whether respiratory rhythmic mechanisms differ between species with different abilities to autoresuscitate from hypothermic respiratory arrest. Based on our overarching hypothesis, the respiratory network of hamsters, a species capable of autoresuscitation from hypothermic respiratory arrest, should be more excitable due to a greater reliance on RIL- or FFA-sensitive mechanisms than rats. We predicted that blocking RIL- and FFA-sensitive mechanisms would have a profound effect on respiratory output in hamsters.

As predicted, RIL profoundly affected respiratory activity in hamsters, reducing both phrenic frequency and burst amplitude (Fig. 3). In fact, application of a low concentration of RIL (1–2 μM) yielded a 50% reduction in burst frequency in weaned hamsters, while in age-matched weaned rats, even concentrations up to 20 μM RIL, had no effect on respiratory frequency. The effect of RIL on hamster preparations is consistent with our earlier study (14) on the isolated brainstem-spinal cord preparation in early postnatal development (0–4 PND) and suggests this difference exists at birth. The effect of RIL was not due to inadequate perfusion of the in situ hamster preparations, as all the preparations displayed the hallmarks of viable and stable preparations including a three-phase respiratory rhythm (Fig. 1; Ref. 34) and maintained ramping phrenic discharge until respiratory rhythm ceased (Fig. 2). In addition, time and vehicle controls maintained respiratory output over similar time courses. However, systemic administration of drug precludes identification of the site of action of RIL. Thus the locus within the respiratory network that confers greater sensitivity to RIL in hamsters remains to be determined.

Inconsistent with our hypothesis, hamsters did not require FFA-sensitive mechanisms for the production of respiratory activity. In addition, contrary to our expectation, coapplication of FFA with RIL resulted in maintenance of respiratory activity even at 20 μM RIL (Fig. 7). This result was in stark contrast to the effect of RIL alone where respiratory activity was completely abolished at RIL >14 μM and is in opposition to the additive effects of the drugs in rats. At the concentrations of FFA used in the present study (2.5–25 μM), FFA would also stimulate large conductance Ca2+-activated K+ channels (22), resulting in an increase in whole cell conductance that may counteract the effects of RIL. The negative interaction between FFA and RIL in hamsters could be occurring within the same population of neurons or within reciprocally inhibiting neurons within the same locus. Alternatively, FFA could have been acting on an upstream region that provides drive to the respiratory network and this effect could have been unmasked when respiratory drive was lowered by blockade of RIL-sensitive...
mechanisms. From the current data, we cannot determine the exact mechanisms or regions where the interaction between FFA and RIL might be occurring, although our data strongly indicate a species-dependent difference in RIL- and FFA-sensitive mechanisms between hamsters and rats.

Hypercapnic/acidotic stimulation had no effect on basal respiratory activity in hamster preparations and did not alter the response to RIL + FFA administration. This is in contrast to results in age-matched rats. Under normoxic/normocapnic conditions, hamsters in vivo have a PCO$_2$ range of 41–52 Torr (31,
50, 51) and a blunted hypercapnic ventilatory response (51). The fossorial lifestyle of hamsters may have led to these physiological adaptations to hypercapnic conditions. Thus the change in PCO2 in the perfusate from 5% CO2 to 7% CO2 may have been insufficient to evoke chemosensory stimulation in the in situ hamster preparation. However, the change in PCO2 also resulted in acidification of the perfusate buffer. This acidosis also had no effect on basal respiratory rhythm and did not alter the effect of RIL + FFA. This suggests that hamsters may also have an altered response to respiratory acidosis compared with rats.

**Physiological Significance**

Data from the present study indicate that respiratory rhythmogenesis in P12–14 rats is more dependent on excitation RIL-sensitive and FFA-sensitive mechanisms than preparations from either younger (neonatal) or older (weaned) rats. This may reflect the changing balance between excitatory and inhibitory mechanisms that occurs during a critical developmental period around this age (53) associated with fluctuations in neurochemical and receptor expression (28, 52, 53) and alterations in physiological responses to respiratory challenges (26, 27, 41). Our data are certainly consistent with the hypothesis of a critical developmental period at the end of the second postnatal week, although, the potential roles of RIL-sensitive and FFA-sensitive mechanisms in the changing physiological responses to respiratory challenges at this age remain to be determined.

The data from the present study and our earlier work (14) indicate a number of fundamental differences in the respiratory rhythmogenic mechanisms of hamsters and rats. First, respiratory rhythmogenesis in hamsters is more reliant on RIL-rhythmogenic mechanisms of hamsters and rats. First, respiratory rhythmogenesis in P12–14 rats is more dependent on excitatory and inhibitory mechanisms that might be involved in autoresuscitation from hypothermic respiratory arrest remains to be elucidated.

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

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