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Abrupt changes in pentobarbital sensitivity in preBötzing complex region, hypoglossal motor nucleus, nucleus *tractus solitarius*, and cortex during rat transitional period (P10–P15)

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Abstract

On postnatal days P10–P15 in rat medulla, neurotransmitter receptor subunit composition shifts towards a more mature phenotype. Since medullary GABA_ARs regulate cardiorespiratory function, abrupt alterations in GABAergic synaptic inhibition could disrupt homeostasis. We hypothesized that GABA_ARs on medullary neurons become more resistant to positive allosteric modulation during P10–P15. Medullary and cortical slices from P10–P20 rats were used to record spontaneous action potentials in pre-Böttinger Complex (preBötC-region), hypoglossal (XII) motor nucleus, *nucleus tractus solitarius* (NTS), and cortex during exposure to pentobarbital (positive allosteric modulator of GABA_ARs). On P14, pentobarbital resistance abruptly increased in preBötC-region and decreased in NTS, but these changes in pentobarbital resistance were not present on P15. Pentobarbital resistance decreased in XII motor nucleus during P11–P15 with a nadir at P14. Abrupt changes in pentobarbital resistance indicate changes in GABAergic receptor composition and function that may compensate for potential increased GABAergic inhibition and respiratory depression that occurs during this key developmental transitional period.

1. INTRODUCTION

GABAergic synaptic transmission plays a key role in the respiratory control network and GABA_ARs are widely expressed on respiratory neurons. GABA_ARs are responsible for fast, synaptic inhibition and also mediate neuronal excitability with a tonic inward inhibitory current. In preBötC neurons, GABA_ARs modulate neuronal excitability and regulate respiratory rhythm generation and pattern (Bongianni et al., 2010; Janczewski et al., 2013; Shao and Feldman, 1997). Also, tonic GABAergic input can constrain the firing rate of medullary respiratory neurons by up to 50% (Zuperku and McCrimmon, 2002), and altered

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GABAergic signaling can significantly disrupt respiratory output (Koshiya and Guyenet, 1996; Paton and Richter, 1995).

There is a “transitional period” in respiratory motor control development during postnatal days P10–P15 in rats that is characterized by abrupt changes towards the adult phenotype in receptor subunit composition and neurotransmitter concentrations in neurons within preBötC (generates inspiratory rhythm), XII motor nucleus (regulates upper airway patency), and NTS (integrates cardiorespiratory sensory input; Liu and Wong-Riley, 2005; Wong-Riley and Liu, 2008). Although “critical period” was used by Wong-Riley and colleagues in their seminal studies of development (Gao *et al.*, 2011; Liu *et al.*, 2006, 2009; Liu and Wong-Riley, 2002, 2004, 2005, 2006, 2010; Wong-Riley and Liu, 2008; Wong-Riley *et al.*, 2013), we prefer to use the term “transitional period” because “critical period” is more often used in neuroscience to define a specific developmental period whereby a perturbation (*e.g.*, sensory deprivation) causes long-lasting changes in neural function (*e.g.*, Hensch and Bilimoria, 2012). The rat transitional period in respiratory control development at P10–P15 represents a progression towards expressing the adult phenotype in receptor subunit composition and neurotransmitter concentrations, but it also represents a potential period of increased vulnerability for maintaining blood-gas homeostasis.

During this period, accumulating evidence suggests there is a net increase in neuronal inhibition. GABAergic inhibition is increased and excitatory signaling is decreased in the XII motor nucleus (Gao *et al.*, 2011). Similarly, in all three regions, glutamate and NMDAR1 decrease, whereas GABA, GABA_BR, glycine receptors, and GluR2 increase at P12 (Liu and Wong-Riley 2002, 2005, 2010). With respect to GABA_ARs, subunit composition switches from including predominately alpha-3 subunits (immature phenotype) to alpha-1 subunits (mature phenotype) on postnatal day 12 in preBötC and NTS neurons, (Liu and Wong-Riley, 2004; 2006). Functionally, these changes may contribute to decreased neuronal excitability, and potentially cause the blunted hypoxic and hypercapnic ventilatory responses in found in P12–P15 rats (Holley *et al.*, 2012; Liu *et al.*, 2006, 2009; Wong-Riley and Liu, 2008). Taken together, these studies suggest that neuronal inhibition is relatively high during the second postnatal week. Despite the obvious importance of maintaining appropriate respiratory control during the transitional period, underlying mechanisms that may potentially compensate for increased inhibition are poorly understood and have received little attention.

Recently, our group identified a compensatory mechanism which likely contributes to respiratory control stability. Specifically, GABA_ARs become insensitive to pentobarbital during hibernation (Hengen *et al.*, 2009, 2011) and pregnancy (Hengen *et al.*, 2012). This response appears to be selective for respiratory-related medullary regions, such as ventral respiratory group and NTS, since cortical neurons remain highly sensitive to pentobarbital during both conditions (Hengen *et al.*, 2009, 2011, 2012). Since there is potentially widespread decreased excitability in the rat medulla during P10–P15, we hypothesized that respiratory-related medullary regions would increase resistance to positive allosteric modulation to maintain appropriate neuronal excitability (*i.e.*, decrease impact of inhibition).

To address this hypothesis, spontaneous neuronal action potentials were recorded in preBötC-region, XII motor nucleus, NTS, and cortex (non-respiratory control) in acutely isolated brain slices from P10–P20 rats using extracellular silicon multichannel electrodes. To pharmacologically assay for resistance to positive allosteric modulation, slices were exposed to pentobarbital and the subsequent decrease in spontaneous action potential firing was quantified. We found that there were complex changes in pentobarbital-resistance that were region- and age-dependent in medulla, but not cortex. Preliminary data were reported previously (Turner and Johnson, 2012).

2. METHODS

2.1 Electrophysiological brain slice recordings in vitro

All experimental procedures followed NIH guidelines and this study was approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. A total of 94 rats at postnatal ages P10–P20 were used for brain slice recordings. Rats were anesthetized with 5% isoflurane (O₂ balance) until the toe-pinch response was abolished. Brains were removed, and medullary and cortical slices (400 µm thick) were cut in ice-cold artificial cerebrospinal fluid (aCSF) solution with a vibrating microtome (Campden Instruments, Lafayette, IN, USA). A series of 3–4 medullary slices used for recording contained the preBötC-region (ventrolateral to the compact *nucleus ambiguus*), XII motor nucleus, and NTS (Figs. 1A–C). Cortical slices contained primary motor and somatosensory areas (Fig. 1D). Slices were placed into an interface recording chamber (Warner Instruments, Hamden, CT, USA) and subfused with aCSF solution (8 ml/min), whose composition was (in mM): 120 NaCl, 26 NaHCO₃, 20 glucose, 2.0 MgSO₄, 1.0 CaCl₂, 1.25 Na₂HPO₄, 7.0 KCl. The KCl concentration was elevated to increase yield of neuronal recordings. Slices were maintained at 37°C by an automated temperature controller (Harvard Apparatus, Holliston, MA, USA). Spontaneous neuronal activity was recorded from preBötC-region, XII motor nucleus, NTS, and cortical neurons using four 16-channel extracellular silicon electrode arrays (model a4x4-3mm100-177, Neuronexus, Ann Arbor, MI, USA). Arrays were composed of four shanks with four recording sites per shank. The distance between each shank was 125 µm, the distance between each recording site was 75 µm, and each individual recording site had a diameter of 15 µm.

2.2 Experimental Protocol

Slices were allowed to equilibrate in aCSF solution with electrodes inserted for 60–90 min before recording baseline activity for 30 min. Afterwards, 200 and 300 µM sodium pentobarbital (Fort Dodge Animal Health, Fort Dodge, Iowa, USA) were sequentially bath-applied to the slices for 45 min each to determine neuronal sensitivity to pentobarbital. The rationale for these concentrations is based on extensive previous work (Hengen et al., 2009; 2012). The pentobarbital concentration sufficient to decrease neuronal firing rates *in vitro* is dependent on overall neuronal excitability in the slice, which is modulated by bath [K⁺]. The goal was to find a balance between the need to increase neuronal yield (by increasing [K⁺]) and demonstrate positive allosteric modulation by pentobarbital. Our experience demonstrates that larger pentobarbital concentrations are required to decrease neuronal firing when bath [K⁺] is increased (Hengen et al., 2009). For this study, bath [K⁺] was

increased slightly above normal ($[K^+] = 3\text{--}5\text{ mM}$) to 7.0 mM and sequential increasing pentobarbital concentrations ($200\text{ }\mu\text{M}$ and $300\text{ }\mu\text{M}$) were tested. This strategy allowed us to: (1) test pentobarbital concentrations that were sufficient to decrease neuronal firing rates in control rats; (2) compare our present results as much as possible with previous results; and (3) minimize animal use by giving the two concentrations sequentially in the same experiment. During the last 30 min of many experiments, muscimol ($20\text{ }\mu\text{M}$, GABA_A receptor agonist, R&D Systems, Minneapolis, MN, USA) was bath-applied to the slices to directly activate GABA_ARs and confirm their presence, especially in neurons that were pentobarbital-resistant.

2.3 Electrophysiological data analysis

Raw extracellular recordings of spontaneous action potentials were processed as described previously (Hengen et al., 2009, 2012). Individual neurons were identified and separated using Principal Component Analysis (Adamos et al., 2008). To group waveforms associated with an individual neuron, all waveforms were projected into multi-dimensional space spanned by the three eigenvectors with the largest associated eigenvalues. The Klustakwik unsupervised clustering algorithm (Harris et al., 2000) was used to identify waveform clusters, assumed to correspond to individual neurons. Neuronal activity was averaged in 1.0-min bins throughout each experiment and normalized to the mean firing rate during a 60-min baseline recording prior to drug application. Individual neurons that were recorded on multiple, adjacent channels were counted only once. Neuronal activity was averaged in 5-min bins and normalized to the mean firing rate during the 30-min baseline recording prior to drug application. Neuronal waveforms were discarded from analysis if any one of the following criteria were met: mean baseline firing rate was $<0.01\text{ Hz}$, absence of action potentials for >10 consecutive min during the baseline period, or there was a consistently decreasing firing rate during baseline to $<50\%$ of the normalized value. Individual bins were discarded if the absolute firing rate was $>500\text{ Hz}$, or if traces exhibited evidence of mechanical disturbances (i.e. normalized firing rate increased and then decreased more than 50 standard deviations from the baseline mean in $<3\text{ min}$). Based on these criteria, 9.3% of waveforms and 0.1% of data bins were discarded.

2.4 Statistical analysis

For statistical comparison, the normalized firing rates were averaged across all neurons within a brain region and age during the last 15 min of drug application. To test for significant differences within a brain region over each postnatal day from P10–P15 and P20, normalized data were analyzed using the Kruskal-Wallis non-parametric one-way ANOVA with Dunn's post-hoc analysis in Sigma Stat software (Jandel Scientific Software, San Rafael, CA, USA). When appropriate, data were log transformed to meet normality and/or equal variance assumptions. To test for abrupt, day-by-day changes within a single brain region, data were compared to the previous, adjacent day using Mann-Whitney Rank Sum t-Tests in Sigma Stat software. This method was used extensively by Wong-Riley and colleagues (e.g., t-test between P10 and P11, P11 and P12, *etc.*). $P<0.05$ was considered statistically significant.

3. RESULTS

3.1 Time control experiments

To confirm that spontaneous neuronal activity remained stable for the duration of our experiments, slices from P10–P15 and P20 rats were subfused for 4 h in standard aCSF solution. Table 1 shows the distribution of neurons ($n=707$) recorded for time control experiments. Neurons from all brain regions produced spontaneous action potentials for 150 min. Data from each brain region were pooled across ages from P10–P15 because all groups produced unaltered activity ($P>0.05$). At the end of time control experiment (150 min time-point), average neuronal firing rates for medullary neurons were $124 \pm 8\%$ (preBötC-region), $119 \pm 8\%$ (XII motor nucleus), and $113 \pm 6\%$ (NTS) relative to baseline (Fig. 1E). The average neuronal firing rates for cortical neurons was unaltered with time ($107 \pm 3\%$ relative to baseline at the 150-min time point), although there was increased variability (Fig. 1F). Data from P20 rats were not pooled along with data from P10–P15 rats because P20 was outside the P10–P15 period. Similar to data from P10–P15 rats, data from P20 rats showed that average neuronal firing rates were $96 \pm 17\%$ (preBötC-region), $115 \pm 12\%$ (XII motor nucleus), $122 \pm 9\%$ (NTS), and $119 \pm 11\%$ (cortex) of baseline at the end of time control experiments ($P>0.05$ for comparisons between P10–P15 and P20 time control data; data not shown). The GABA_AR agonist muscimol (20 μ M) was applied during the final 15 min in a subset of time control experiments to test determine what percentage of neurons expressed GABA_ARs. Accordingly, muscimol application decreased average neuronal firing rates by $>40\%$ of baseline values in 265 of 270 neurons (98%). These data showed that nearly all recorded neurons expressed functional GABA_ARs.

3.2 Pentobarbital resistance abruptly increased in PBC-region neurons at P14

To compare steady-state pentobarbital responses, the last 15 min of the spontaneous neuronal firing rate (normalized to baseline) was averaged for each age at 200 and 300 μ M pentobarbital for all four regions (Figs. 3–6). Table 2 shows the number of recorded neurons ($n=1,118$) at each age in all four regions. In the preBötC-region, pentobarbital application decreased spontaneous firing rates similarly across all ages except at P14 when an increase in pentobarbital resistance was observed ($p<0.05$ for drug effect; Fig. 2). During the last 15 min of the 200 μ M pentobarbital application, the average preBötC-region neuron firing rate was 61–73% of baseline in P10–P13 rats, increased to $133 \pm 22\%$ in P14 rats ($p<0.05$), and returned to 63–69% in P15 and P20 rats ($p=0.002$ for age effect; Fig. 3A). To examine the distribution of pentobarbital responses as a function of age, preBötC-region neurons were classified based on the average firing rates from the last 15 min of the 200 μ M pentobarbital application. Neurons were classified as sensitive ($<40\%$ of baseline), intermediate (40–80% of baseline) or resistant ($>80\%$ of baseline). The percentage of resistant preBötC-region neurons increased from 16% (P11) to 39% (P13) and peaked at 47% (P14) before decreasing to 32% (P15) and 35% (P20) (Fig. 3B). Pentobarbital-sensitive preBötC-region neurons abruptly decreased to 22% of neurons at P14 compared to 39–42% of neurons at P13 and P15 (Fig. 3B).

Similarly, during the last 15 min of 300 μ M pentobarbital, average firing rates in preBötC-region neurons abruptly increased to $89 \pm 21\%$ of baseline at P14 ($p<0.05$ vs. P15)

compared to 23–41% on the other postnatal days ($p=0.005$ for age effect; Fig. 3C). There was an increase in average firing rate from $23 \pm 4\%$ of baseline at P11 to $41 \pm 7\%$ at P12 ($p<0.05$; Fig. 3C). The average firing rate increased from $41 \pm 7\%$ at P13 to $89 \pm 21\%$ at P14, but this was not significant ($p=0.21$). The average firing rate abruptly decreased the next day to $35 \pm 8\%$ at P15 ($p=0.014$). Categorizing preBötC-region neuron average firing rates during the last 15 min of 300 μM pentobarbital showed that the percent of pentobarbital-resistant neurons abruptly increased from 14% (P13) to 33% (P14), followed by a decrease to 19% at P15 (Fig. 3D). In contrast, 5% of neurons from P10 rats were pentobarbital-resistant and 96% were pentobarbital-sensitive with no neurons having intermediate pentobarbital sensitivity (Fig. 3D). Thus, preBötC-region neurons had increased sensitivity to pentobarbital at P11 and increased resistance to pentobarbital at P14. Overall, the pentobarbital responses were similar with respect to age at both 200 μM and 300 μM .

3.3 Pentobarbital resistance decreased in XII motor nucleus – minimum resistance at P14

For neurons in the XII motor nucleus with the 200 μM pentobarbital application, pentobarbital resistance decreased and increased in an age-dependent manner (Fig. 4A). XII neuron average firing rates increased from day P12 ($41 \pm 12\%$) to day P13 ($77 \pm 13\%$; $p<0.05$), and decreased on day P14 ($61 \pm 8\%$; $p<0.05$ compared to P13; Fig. 4A). There was a second increase in XII neuron firing rates from day P15 ($56 \pm 8\%$) to day P20 ($84 \pm 10\%$; $p<0.001$ comparing P15 with P20; $p<0.001$ for age effect; Fig. 4A). In general, there was a correlation between the percentage of pentobarbital-resistant neurons and steady-state firing rate for the 200 μM pentobarbital application (Fig. 4B). The percentage of pentobarbital-resistant XII neurons was 37% at P10, 12% at P12, and 51% resistant neurons at P20 (Fig. 4B). Accordingly, the percentage of pentobarbital-sensitive XII motoneurons ranged from 81% of neurons at P12 to 22% of neurons at P20.

However, during the 300 μM pentobarbital application, pentobarbital resistance decreased with age to a nadir at P14 while resistance at P10 and P20 were equivalent (Fig. 4C). XII neuron average firing rates progressively decreased from $61 \pm 11\%$ (P10) to $15 \pm 3\%$ (P14) before increasing up to $53 \pm 6\%$ (P20) of baseline ($p<0.001$ for age effect; Fig. 4C). Average firing rates in P11–P15 XII motoneurons were lower compared firing rates in P20 rats ($p<0.05$; Fig. 4C). The percentage of pentobarbital-resistant XII motoneurons was 21–22% at P10 and P20, but only 3% of neurons were resistant at P13 and P14 (Fig. 4D). Accordingly, the percentage of pentobarbital-sensitive XII motoneurons increased from 54% at P10 to 81–88% at P12–P14 with very few intermediate resistant neurons (Fig. 4D). The profile of pentobarbital responses at 200 μM pentobarbital (Figs. 4A, 4B) was different compared to the profile at 300 μM pentobarbital (Figs. 4C, 4D). The data from the 300 μM pentobarbital application suggest that there was a dramatic decrease in pentobarbital resistance in XII neurons at P13 and P14.

3.4 Pentobarbital resistance in NTS neurons decreased abruptly at P14

In NTS, pentobarbital resistance was relatively constant for the 200 μM pentobarbital application during the P10–P20 period with NTS neuron firing rates at 52–74% of baseline ($p=0.349$ for age effect; Fig. 5A). The only change was a modest increase from $52 \pm 11\%$

(P15) to $65 \pm 7\%$ (P20) of baseline ($p=0.015$; Fig. 5A). The percentages of pentobarbital-resistant, intermediate, and pentobarbital-sensitive NTS neurons were variable and did not show any obvious trends (Fig. 5B). The percentage of pentobarbital-resistant NTS neurons was lowest with 11% at P11 and maximal with 35% at P20 (Fig. 5B).

For the 300 μM pentobarbital application, NTS neuron average firing rates were relatively unchanged except for when NTS neuron firing rates decreased from $54 \pm 7\%$ at P13 to $23 \pm 8\%$ at P14 ($p<0.05$), but then increased back to $37 \pm 8\%$ at P15 ($p<0.05$; $p<0.001$ for age effect; Fig. 5C). The percentage of pentobarbital-resistant NTS neurons was unremarkable except for an abrupt decrease to 5% at P14 with a corresponding maximum of 83% of pentobarbital-sensitive neurons (Fig. 5D). Thus, the responses of NTS neurons were similar for both the 200 μM and 300 μM pentobarbital applications, with the 300 μM application revealing a decrease in pentobarbital resistance at P14, similar to the decrease in XII neurons at P14.

3.5 Pentobarbital resistance in cortical neurons abruptly decreased at P12

Cortical neurons were generally more sensitive to pentobarbital than medullary neurons with an abrupt decrease in pentobarbital resistance at P12. For the 200 μM pentobarbital application, cortical neuron average firing rates ranged from 32–68% of baseline, except at P12 where the firing rate decreased to $18 \pm 3\%$ before returning to $38 \pm 5\%$ at P13 ($p<0.004$ compared to P13; $p=0.019$ for age effect; Fig. 6A). There were no pentobarbital-resistant cortical neurons at P12 and 89% of cortical neurons were pentobarbital-sensitive (Fig. 6B). For P20 rats, 30% of neurons were pentobarbital-resistant and 55% were pentobarbital-sensitive (Fig. 6B). For the 300 μM pentobarbital application, cortical neuron average firing rates were lowest at P12 ($14 \pm 2\%$) and P20 ($15 \pm 8\%$) compared to 21–30% of baseline on the other postnatal days ($p<0.05$ at P12 and P20; $p=0.003$ for age effect; Fig. 6C). Accordingly, at P12 and P20, there were no pentobarbital-resistant cortical neurons and 92–93% of neurons were pentobarbital-sensitive (Fig. 6D). The percentage of pentobarbital-resistant cortical neurons was very low at the other ages and ranged between 1–8%. Thus, cortical neurons were more sensitive to pentobarbital than medullary neurons, especially at P12.

3.6 Muscimol-resistant neurons and baseline firing rates within age groups

Only 20 neurons did not decrease their firing rates during muscimol application. These resistant neurons were not clustered in any age or region (Table 3). With respect to baseline firing rates, there were no shifts or trends with respect to age for each region (Table 4). PBC neurons did not have any alterations in baseline firing rate with respect to age ($p=0.83$). P20 XII motoneuron baseline firing rates were decreased compared to P15 XII motoneurons ($p<0.05$), and P10 and P20 NTS neuron baseline firing rates were decreased compared to P14 NTS neurons ($p<0.05$). P10 cortical neuron baseline firing rates were decreased compared to P14 cortical neurons ($p<0.05$). None of these changes correlated with age- and region-dependent changes in pentobarbital sensitivity.

4. DISCUSSION

The main finding was that the resistance of respiratory medullary neurons to the GABAergic modulator pentobarbital abruptly changes over the P10–P15 postnatal period. Contrary to our original hypothesis, pentobarbital resistance of medullary neurons did not uniformly increase across respiratory-related brain regions. Instead, there were abrupt increases (preBötC-region) or decreases (NTS, XII motor nucleus) at P14. Similar to our group's previously published work (Hengen et al., 2009), we found that 300 μ M rather than 200 μ M pentobarbital was the most effective dose for detecting differences in pentobarbital resistance. The present study suggests that potency of GABAergic modulators is dynamically regulated in an age- and region-dependent manner during development. These findings also suggest that P14 (rather than the P11–P12 transition) is an important day in rat neural development with respect to GABA_AR function. Changing sensitivity to positive allosteric modulation within GABA_AR at P14 may contribute, in part, to physiological changes in breathing known to occur during the second postnatal week.

4.1 Increased neuronal inhibition during the transitional period in respiratory motor control

During the rat transitional period (P10–P15) there are a variety of profound changes in behavior, physiological responses and neurophysiological variables. Notable examples include eye and ear opening, development of normal sleep patterns, reorganization of the hypoxic ventilatory response, and rapid myelination in the CNS (Kubin and Volgin, 2008). With respect to respiratory control development, the balance of neuronal excitation versus inhibition shifts towards increased inhibition. Indeed, during the transitional period, glutamate concentrations and NMDA receptor subunits NR1 and NR2A expression decrease, while GABA, GABA_B receptor, and glycine receptor expression increases in preBötC, XII motor nucleus, and NTS (Liu and Wong-Riley, 2002, 2005, 2010). Additionally, chloride transporters transition to the adult phenotype from mainly expressing NKCC1 (promotes high intracellular chloride levels) at birth to predominately expressing KCC2 (promotes low intracellular chloride levels and hyperpolarizing GABA currents) at P12 in preBötC, XII motor nucleus, and NTS (Liu and Wong-Riley, 2012). Electrophysiological data also suggest increased inhibition in XII motor nucleus neurons at P12–13 because: (1) amplitude and frequency of spontaneous EPSCs decreases while amplitude and frequency of spontaneous IPSCs increases; and (2) amplitude and charge transfer of mEPSCs is reduced while the amplitude, frequency and charge transfer of mIPSCs is increased (Gao *et al.*, 2011). With respect to GABA_ARs, the subunit composition switches from predominately expressing α -3 subunits to predominately expressing α -1 subunits in preBötC and NTS neurons (Liu and Wong-Riley, 2004, 2006), which likely improves the efficacy of inhibition by decreasing channel decay time (Bosman *et al.*, 2002). These findings suggest that inhibitory neurotransmission is more dominant within the medullary respiratory control network by P12. Accordingly, we hypothesized that pentobarbital resistance would increase in medullary GABA_ARs during P10–P15 to compensate for the increase in inhibitory neurotransmission. Instead, our data show that pentobarbital resistance is dynamically regulated in an age- and region-dependent manner throughout the medulla.

Another surprising finding from this study was that abrupt increases or decreases in pentobarbital resistance that we observed occurred mainly at P14, rather than at P11–P12 as elegantly demonstrated repeatedly by Wong-Riley and colleagues (Gao *et al.*, 2011; Liu *et al.*, 2006, 2009; Liu and Wong-Riley, 2002, 2004, 2005, 2006, 2010; Wong-Riley and Liu, 2008, Wong-Riley *et al.*, 2013). We speculate that the abrupt changes in the medulla at P11–P12 may initiate a cascade of events that lead to further changes in the next two days that may compromise breathing. This hypothesis is based on studies in rats showing alterations in breathing and hypoxic responses between P12–P14. During normoxic breathing, respiratory frequency increases from P0 to a peak at P13 before gradually declining (Liu *et al.*, 2006). During acute hypoxia, the ventilatory response is weakest in P13 animals, and is significantly lower from P12–P14 compared to the rest of the first three postnatal weeks (Liu *et al.*, 2006). At P13, the combination of relatively low metabolism, and low ratios of ventilation/O₂ consumption and ventilation/CO₂ production are hypothesized to be unique for the entire first 21 postnatal days and put the rats at risk during a severe, prolonged bout of hypoxia (Liu *et al.*, 2009). Likewise, in a different laboratory, P12–13 male rats were found to have lower ventilation during hypercapnia (Holley *et al.*, 2012). These changes suggest that the abrupt rearrangement of receptor subunit expression and neurotransmitter concentrations establishes conditions that predispose rats to a vulnerable day (P13) in respiratory motor control. Accordingly, the changes in pentobarbital resistance that we observed in the preBötC-region may be part of a compensatory mechanism to increase excitability and maintain breathing frequency. In contrast, the reason for the decrease in pentobarbital resistance in XII motor nucleus and NTS neurons is not clear. Taken together, these data suggest that the transitional period in rats extends from P10 to P15, and that complex poorly understood mechanisms regulate the ability of GABA_AR to respond to positive allosteric modulators.

4.2 Potential mechanisms underlying changes in resistance to positive allosteric modulation

The subunit composition of GABA_ARs mediate GABA affinity, efficacy, channel gating properties (Gringrich *et al.*, 1995; Lavoie *et al.*, 1997; Kash *et al.*, 2003), and sensitivity to positive allosteric modulators (Olsen, 1998). Thus, reconfiguring the subunit composition to reflect a different GABA_AR subtype will modulate functional properties of the receptor. The most typically expressed GABA_AR is composed of two alpha, two beta and one gamma subunit (Bonnert *et al.*, 1999). Inclusions of less commonly expressed subunits such as delta or epsilon increase or decrease the receptor's sensitivity to positive allosteric modulation, respectively (Davies *et al.*, 1997; Irnaten *et al.*, 2002; Wagner *et al.*, 2005; Olsen *et al.*, 2007; Hevers and Lüddens, 1998). With respect to our results, we hypothesize that GABA_AR shift subtypes to incorporate the rare epsilon subunit to provide insensitivity to positive allosteric modulation. Inclusion of epsilon subunits into GABA_ARs is the best explanation for our results for the following reasons. First, epsilon subunits are the only known subunit to confer insensitivity to GABA_AR positive allosteric modulators such as pentobarbital (Irnaten *et al.*, 2002; Wagner *et al.*, 2005) and allopregnanolone (Davies *et al.*, 1997). Second, simply decreasing the inclusion of delta subunits (heighten sensitivity to positive allosteric modulation) (Olsen *et al.*, 2007; Hevers and Lüddens, 1998) does not explain our results because standard GABA_ARs (without delta subunits) are also sensitive to positive allosteric

modulators. Additionally, delta subunit mRNA is very low in the preBötC-region, XII motor nucleus, and NTS in P10–P15 rats (S.M. Turner and S.M. Johnson, unpublished observations) and no delta subunit immunoreactivity was observed in the ventral respiratory column in 13-lined ground squirrels (Hengen et al., 2011). These data suggest that delta subunits expression is very low in key respiratory-related medullary regions and that a decrease in delta subunit inclusion in GABA_ARs is highly unlikely to account for changes in pentobarbital resistance observed in this study. Third, our results are similar to our previously published reports of increased epsilon subunit expression during hibernation and pregnancy. During the torpor phase of hibernation in 13-lined ground squirrels, cortical neurons are electrically silent, yet the brainstem continues to regulate cardiorespiratory function (Drew et al., 2001, 2007). Epsilon subunits appear to help maintain cardiorespiratory function during the hibernation cycle because NTS and ventrolateral medullary neurons have increased resistance to pentobarbital in hibernating squirrels compared to summer active squirrels (Hengen et al., 2009). Further, neurons within the ventral respiratory column nearly double epsilon subunit immunoreactivity during hibernation (Hengen et al., 2011). In contrast, cortical neurons are electrically silent during torpor, remain sensitive to pentobarbital, and do not increase epsilon subunit immunoreactivity (Hengen et al., 2009; 2011). Likewise, during late pregnancy in rats, when central allopregnanolone concentrations increase 3–4 fold (Concas et al., 1998), respiratory motor output on the phrenic nerve is maintained longer during sequential pentobarbital injections compared to non-pregnant female rats (Hengen et al., 2012). Respiratory frequency is preserved rather than phrenic burst amplitude, suggesting the preBötC neurons increase epsilon subunit expression. Consistent with this hypothesis, preBötC-region neurons from pregnant rats are more resistant to pentobarbital and have increased epsilon subunit immunoreactivity compared to non-pregnant female and male rats (Hengen et al., 2012). Thus, evidence from these two experimental models suggests that epsilon subunit expression increases in respiratory-related medullary regions to protect breathing and cardiovascular regulation from excessive inhibition due to positive allosteric modulation of GABA_ARs.

The mechanisms regulating subunit expression and inclusion in GABA_ARs are still poorly understood and require further investigation into what factors regulate their transcription, translation, and insertion into plasma membrane GABA_ARs. With respect to this study, quantifying potentially small changes in GABA_AR epsilon subunit levels in very specific brain regions using semi-quantitative immunohistochemistry or western blot techniques may not reveal whether the epsilon subunits were included into functional GABA_ARs in the plasma membrane to alter the capacity for allosteric modulation of GABA_ARs. Thus, demonstrating anatomically that epsilon subunit expression is increased in preBötC-region neurons at P14, or that epsilon expression is decreased in XII motor nucleus or NTS neurons at P14 is beyond the scope of this manuscript. One potential problem is that epsilon subunit protein may be altered in only a subpopulation of neurons (sufficient to change the average response to pentobarbital). In addition, there may be similar amounts of epsilon subunit protein at P13 and P14, but the epsilon subunit protein may undergo trafficking to or from the plasma membrane at P14 to confer increased or decreased pentobarbital resistance, respectively. Thus, we assert that the use of electrophysiological techniques to quantify

pentobarbital resistance is a more sensitive method for detecting subtle, but important changes in GABA_AR subunit function and composition in medullary neurons.

4.3 Abrupt decrease in pentobarbital resistance in cortical neurons

In this study, cortical neurons were generally highly sensitive to pentobarbital during the 300 μ M pentobarbital application, but abruptly became even more sensitive to pentobarbital at P12. The high sensitivity to pentobarbital may be due to decreased epsilon expression, but it is possible that heightened pentobarbital sensitivity may be due to altered expression of other GABA_AR subunits, such as delta subunits, which are highly sensitive to positive allosteric modulation (Olsen et al., 2007; Hevers and Lüddens, 1998). The hypothesis that delta subunits are increased in cortical neurons of P12 rats is supported by electrophysiological data showing a significant decrease in GABA_AR modulation by flunitrazepam in P12 rats compared to P7 rats (Grobin and Morrow, 2001). This finding supports an increase in delta subunit incorporation because benzodiazepines bind between alpha and gamma subunits to enhance chloride ion influx, however, delta-containing GABA_AR lack gamma subunits (Araujo et al., 1998; Quirk et al., 1995), rendering the receptor insensitive to benzodiazepines. Therefore, decreased sensitivity to a benzodiazepine such as flunitrazepam suggests increased delta subunit inclusion in GABA_ARs. These data, considered along with our results, suggest delta subunit expression may play an important developmental role in cortical neurons while changes epsilon subunit incorporation may be specific to respiratory-related brain regions.

4.5 Physiological and Clinical Significance

The transitional P10–P15 period is well-defined in rats, but not in other mammals, such as humans. Translating neurodevelopmental milestones across species for relative developmental ages is challenging with conflicting reports in the literature. In one study, rat postnatal days P10–15 are correlated with the human postnatal months 2–4 (Ballanyi et al., 2004). In contrast, a detailed analysis of neurodevelopmental events suggests that a P12–13 rat corresponds most closely with a G196 human fetus (i.e., early third trimester; Clancy et al., 2007). In either case, it's important to understand the potential effects of abrupt changes in medullary receptors and neurotransmitters with respect to cardiorespiratory function. If the P10–P15 rat transition period translates most closely to a 2–4 month old human infants, then this time frame correlates with the peak incidence of sudden infant death syndrome (SIDS; Goldberg et al., 1986; Harper and Kinney, 2010). This correlation corresponds with the inability of P12–P14 rats to appropriately respond to hypoxia for a brief time period, and may represent increased vulnerability to SIDS.

On the other hand, if the P10–P15 rat transitional period translates more closely to pre-term infants, then there could be important implications for apnea of prematurity. Human infants born in the early third trimester have often suffer from apnea of prematurity and the insufficient ventilatory response to hypoxia leads to blood oxygen desaturation and bradycardias (Raju et al., 2012; Vergales et al., 2013). However, pre-term infants with apnea of prematurity outgrow the condition by or before full-term gestational age, suggesting a brief period of rapid development and potential instability in respiratory motor control. Thus, determining whether human pre-term or postnatal infants undergo similar abrupt

changes in medullary receptors and neurotransmitters may be an important step in increasing awareness of potential cardiorespiratory instability and pathological breathing conditions.

5. Conclusion

This study showed that medullary neurons abruptly changed their response to positive allosteric modulation of GABA_ARs on P14, which we hypothesize is due to changes in epsilon subunit inclusion into GABA_ARs. The changes observed at P14 may be part of a larger series of developmental processes that begin at P11–P12 when there is a widespread reorganization of receptor subunits and neurotransmitters in the medulla. During the transitional period, there are documented deficiencies in ventilatory responses to hypoxia and hypercapnia, which may reflect a potential vulnerable period with respect to respiratory motor control. The changes in pentobarbital resistance at P14 may contribute to the vulnerability or help compensate for the other changes in the regulation of breathing.

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ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
cNA	compact <i>nucleus ambiguus</i>
GABA _A Rs	GABA _A receptors
NTS	<i>nucleus tractus solitarius</i>
preBötC	pre-Bötzinger complex
VRC	ventral respiratory column
XII	hypoglossal

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Highlights

- Pentobarbital sensitivity changed during P10–p15 in rat medullary and cortical slices.
- Pentobarbital resistance in preBötC-region neurons increased on P14.
- Pentobarbital resistance in hypoglossal nucleus decreased with nadir at P14.
- Pentobarbital resistance in *nucleus tractus solitarius* decreased on P14.
- Abrupt changes may compensate for increased GABAergic inhibition during P11–P14.

Figure 1. Location of recordings in medullary and cortical slices and time control experiments
Silicon multichannel electrodes were positioned in the shaded areas to extracellularly record spontaneous action potentials from preBötC-region neurons (A), NTS and XII motor nucleus neurons (B, C). Cortical neurons were recorded as shown in (D). (E) Pre-BötC-region (black circles), XII motor nucleus (white circles), and NTS neuron (white squares) spontaneous firing rates were unchanged for 150 min. Muscimol (20 μ M) application decreased the firing rates of nearly all neurons, demonstrating that these neurons had functional GABA_ARs. (F) Cortical neuron (black circles) did not change their spontaneous firing rate over 150 min, although there was some increased variability during the 110–150 min period. Muscimol (20 μ M) application decreased the firing rates of nearly all neurons.

Figure 2. Pentobarbital resistance abruptly increased at P14 in preBötC-region neurons

This figure shows the typical experimental protocol whereby slices were exposed sequentially to 200 μ M and 300 μ M pentobarbital (45 min each) before being exposed to muscimol (20 μ M) for 20 min. The muscimol (GABA_AR agonist) application is important because it tests whether pentobarbital resistant neurons express GABA_ARs. For preBötC-region neurons in slices from P10–P13 rats and P15–P20 rats, the average neuronal firing rates were 61–73% of baseline during the end of the 200 μ M pentobarbital application, and 23–47% of baseline during the end of the 300 μ M pentobarbital application. At P14, however, the average firing rate was 122–144% of baseline during the end of the 200 μ M pentobarbital application, and 74–85% of baseline during the end of the 300 μ M pentobarbital application ($p < 0.05$ at P14; $p = 0.002$ for age effect). Symbols: P10 (small white circles), P11 (medium gray circles), P12 (large black circles), P13 (small white triangles), P14 (medium gray triangles), P15 (large black triangles), P20 (white squares), † indicates drug effect $p < 0.05$.

Figure 3. Pentobarbital resistance and distribution of resistant and sensitive preBötC-region neurons

(A) In preBötC-region neurons, average firing rates from P10–P13 rats and P15–P20 rats was 61–73% of baseline during the last 15 min of the 200 μ M pentobarbital application. At P14, the average firing rate abruptly increased to $133 \pm 22\%$ of baseline ($p < 0.05$ at P14; $p = 0.002$ for age effect). (B) Classifying preBötC-region neuron average firing rates from 200 μ M pentobarbital application as sensitive ($< 40\%$ of baseline; white), intermediate (40–80% of baseline; gray) or resistant ($> 80\%$ of baseline; black) showed that the percentage of resistant cells steadily increased from 16% at P11 to 47% at P14. (C) With 300 μ M pentobarbital application, preBötC-region neuron average firing rates abruptly increased to $89 \pm 21\%$ of baseline at P14 ($p < 0.05$) compared to 23–41% of baseline on the other postnatal days ($p = 0.005$ for age effect). (D) With the 300 μ M pentobarbital application, preBötC-region neurons resistant to pentobarbital abruptly increased from 14% of neurons at P13 to 33% of neurons at P14. Symbols: # = different from the day indicated by the horizontal bar ($p < 0.05$ for Dunn's post-hoc analysis), * = different from the previous day ($p < 0.05$ for t-test results).

Figure 4. Pentobarbital resistance decreased in XII motor nucleus neurons

(A) Average firing rates in XII motor nucleus neurons decreased from $72 \pm 10\%$ of baseline at P10 to only 41–49% of baseline at P11–12 before increasing to $77 \pm 13\%$ of baseline at P13 ($p < 0.05$ at P13; $p < 0.001$ for age effect) during the last 15 min of 200 μ M pentobarbital. (B) Classifying XII motor nucleus neuron average firing rates as sensitive (<40% of baseline; white), intermediate (40–80% of baseline; gray) or resistant (>80% of baseline; black) showed that at P10 37% of cells were resistant to pentobarbital. However, the percent resistant neurons decreased to only 12% at P12. (C) During 300 μ M pentobarbital application, XII motor nucleus neuron average firing rates decreased from $61 \pm 11\%$ of baseline at P10 to $15 \pm 3\%$ of baseline at P14 before returning to $53 \pm 6\%$ of baseline at P20 ($p < 0.001$ for age effect). (D) With the 300 μ M pentobarbital application, 22% of neurons were resistant to pentobarbital in P10 and P20 rats. However, the percent resistant neurons decreased to only 3–9% of neurons during P11–P15. Symbols are the same as in Fig. 3.

Figure 5. Pentobarbital resistance decreased at P14 in NTS neurons

(A) During the last 15 min of the 200 μ M pentobarbital application, average firing rates in NTS neurons decreased to 52–74% with no obvious trends ($p=0.349$ for development effect). P15 rats had lower average firing rates at $52 \pm 11\%$ of baseline compared to $65 \pm 7\%$ of baseline in P20 rats ($p<0.05$). (B) Classifying NTS neurons as sensitive (<40% of baseline; white), intermediate (40–80% of baseline; gray) or resistant (>80% of baseline; black) showed that in P11 rats, only 11% of cells were pentobarbital resistant whereas pentobarbital resistance was found in 26% and 35% of cells in P13 and P20 rats, respectively. (C) During the last 15 min of the 300 μ M pentobarbital application, NTS neuron average firing rates decreased to 23–54% of baseline ($p<0.001$ for age effect) with highest average firing rates at $54 \pm 7\%$ of baseline in P13 rats, and the lowest average firing rates at $23 \pm 8\%$ of baseline in P14 rats ($p<0.05$). (D) For the 300 μ M pentobarbital application, 21% of NTS neurons at P13 were resistant, but abruptly decreased to only 5% of NTS neurons at P14.

Figure 6. Pentobarbital resistance abruptly decreased at P12 in cortical neurons

(A) In cortical neurons, 200 μ M pentobarbital application decreased average firing rates to 32–68% of baseline, except at P12 where the average firing rate abruptly decreased to 18% of baseline before returning to 38% of baseline at P13 ($p < 0.004$ at P12; $p = 0.019$ for age effect). (B) Classifying cortical neuron average firing rates during the last 15 min of the 200 μ M pentobarbital application into sensitive (<40% of baseline; white), intermediate (40–80% of baseline; gray) or resistant (>80% of baseline; black) showed that at P12 there were no resistant neurons whereas P20 had the greatest percentage of resistant neurons at 30% of neurons. (C) During 300 μ M pentobarbital application, cortical neuron average firing rates were lowest at P12 and P20 at 14–15% of baseline, respectively, compared to 21–30% of baseline on the other postnatal days ($p < 0.05$ at P12 and P20; $p = 0.003$ for age effect). (D) For the 300 μ M pentobarbital application, there were no resistant neurons at P12 or P20. The percent of pentobarbital resistant neurons was very low compared to medullary neurons. Symbols are the same as in Fig. 3.

Table 1
Numbers of neurons recorded in time control experiments

Spontaneous action potentials were recorded from a total of 270 neurons for time control experiments.

	P10–P15 (n=26 rats)	P20 (n=7 rats)
PBC-region neurons	127	24
XII motoneurons	100	47
NTS neurons	127	57
Cortical neurons	175	50

Table 2
Numbers of neurons recorded in pentobarbital-application experiments

A total of 61 rats (number of rats per age is indicated in the top row under the age header) were used for pentobarbital application experiments to record spontaneous action potentials from 1,118 neurons. The distribution of neuronal recordings is shown for each age and region.

	P10 (n=6)	P11 (n=7)	P12 (n=8)	P13 (n=10)	P14 (n=11)	P15 (n=10)	P20 (n=9)
PBC-region neurons	24	37	31	39	45	31	51
XII motoneurons	41	32	26	36	38	97	46
NTS neurons	34	28	38	47	40	40	31
Cortical neurons	47	56	38	35	24	42	44

Table 3

Number of pentobarbital- and muscimol-resistant neurons

Muscimol sensitivity was tested in a total of 971 neurons during pentobarbital (n=701 neurons) and time control (n=270 neurons) experiments. Only 20 neurons were resistant to muscimol application. The distribution of these cells for each age and region shows that there were no trends for neurons resistant to pentobarbital and muscimol.

	P10	P11	P12	P13	P14	P15	P20
PBC-region neurons	-	1	-	-	-	-	-
XII motoneurons	2	-	1	-	-	2	5
NTS neurons	1	-	-	2	1	1	1
Cortical neurons	2	-	-	-	-	-	1

Table 4
Baseline neuronal firing rates compared by age within each region

Data are shown as mean baseline firing rate (Hz) with the SEM.

	P10	P11	P12	P13	P14	P15	P20
PBC	7.13±1.63	6.24±1.45	5.83±1.39	5.25±0.83	6.92±1.27	7.36±1.25	5.37±0.96
XII	3.60±0.78	3.63±0.78	5.43±1.18	4.66±0.99	5.24±1.11	5.59±0.85	3.31±0.73@
NTS	1.59±0.14*	2.36±0.36	3.18±0.43	3.03±0.55	3.96±0.64	3.19±0.60	2.46±0.60*
Cortex	1.79±0.33*	2.59±0.24	1.88±0.31	2.17±0.22	3.69±0.67	2.72±0.55	4.64±0.99

* p<0.05 compared to P14;

@ p<0.05 compared to P15.