Role of Inhibitory Neurotransmitter Interactions in the Pathogenesis of Neonatal Apnea: Implications for Management

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Apnea of prematurity remains a troublesome clinical problem in the care of low birth weight infants. It may significantly impact the neonatal course of such patients by necessitating the use of assisted ventilation, prolonging their hospitalization, and resulting in a need for xanthine or other pharmacologic intervention. To better understand the physiologic basis for neonatal apnea, the response of respiratory neural output to hypercapnia, hypoxia, and stimulation of laryngeal and other mechanoreceptor-mediated afferents has been extensively studied in both human infants and various animal models. However, maturation of the role of neurotransmitters that mediate these neural pathways at the brainstem is poorly understood. As fetal and early neonatal life are characterized by greater inhibition of respiratory output than in later life, it is tempting to speculate that this period is associated with greater expression of inhibitory versus excitatory neurotransmitters and neuromodulators in respiratory-related neurons. A variety of such substances have been implicated in neonatal respiratory control and some, such as serotonin and adenosine, may have excitatory or inhibitory effects depending on the receptor subtypes activated (Fig 1). Although prostaglandins and endorphins have both been studied for their role as inhibitory neurotransmitters in relation to neonatal respiratory control, the focus of this review will be on gamma aminobutyric acid (GABA), adenosine, and their potential interaction as the major modulators of respiratory neural output in early life.

Part One

GABA in Neonatal Respiratory Control

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system and mediates its effects via fast and slow synaptic transmission. In fast inhibitory transmission, GABA binds to its GABA$_A$ receptor and results in cell hyperpolarization by opening ligand-operated ion channels; slow synaptic transmission is mediated via activation of GABA$_B$ receptors and modulation of downstream signaling pathways. GABA$_A$ receptors consist of five protein subunits; the “mix” of which differs during maturation. While essentially an inhibitor of neural activity in the central nervous system, GABA may produce excitatory responses in the immature brain; however, such excitatory effects have not been clearly observed in relation to neonatal respiratory control. What is the evidence that GABA plays a prominent role in inhibition of respiratory neural output in early life? Such a concept is consistent with the data of Xia and Haddad that GABA$_A$ receptors appear very early in maturation, especially in brainstem regions of the developing rat brain.

Role of GABA in Modulating Hypercapnic Responses

Both prematurity and apnea are associated with impaired ventilatory responses to hypercapnia in human infants, in which the increase in tidal volume is associated with a progressive decrease in frequency due to prolongation of expiratory time. In a series of studies in rat pups and piglets, we sought to test the hypothesis that release of GABA and activation of GABA$_A$ receptors contribute to this phenomenon. In rat pups, we showed that prolongation of expiration and slowing of breathing frequency, in response to hypercapnia, oc-
curred in 5-day-old, but not older (16 days and beyond) animals.\textsuperscript{7} Furthermore, intravenous administration of the GABA\textsubscript{A} receptor blocker, bicuculline, eliminated hypercapnia-induced prolongation of expiration in the youngest animals. This led us to conclude that centrally mediated prolongation of expiration during early postnatal life is mediated via GABAergic inhibition of respiratory timing mechanisms. A similar phenomenon was observed in piglets exposed to hypercapnia, suggesting that this GABAergic effect on respiratory timing is consistently observed in young animals across species.\textsuperscript{8}

These physiologic data are strengthened by our neuroanatomic findings that hypercapnia actually activates GABAergic neurons. In a series of experiments, we employed expression of c-Fos protein, the product of an immediate early gene (c-Fos), to localize CO\textsubscript{2}-activated cells within the piglet medulla oblongata.\textsuperscript{9} This gene is rapidly and transiently expressed following cell activation by different stimuli, including hypercapnia. It should be noted that finding c-Fos expression in a subset of neurons within respiratory-related medullary regions does not prove that these neurons are chemosensitive; however, it does indicate that they are part of the CO\textsubscript{2}-chemosensitive network at that site. After hypercapnic exposure (10% CO\textsubscript{2} for 60 minutes) animals were killed, brains removed and sectioned, and prepared for immunofluorescence. As expected, CO\textsubscript{2} elicited a significant increase in c-Fos-expressing cells when compared with normocapnic controls.

Parvalbumin was then chosen as a marker for GABAergic neurons to determine whether CO\textsubscript{2} exposure elicited c-Fos expression in GABA-containing cells of the medullary network. Previous work has shown that, at all developmental ages, GABA-containing neuronal cell bodies, dendrites, and axonal terminals are parvalbumen-immunoreactive.\textsuperscript{10} Hence, parvalbumen expression can be used to define the organization of GABAergic inhibitory circuits in the brain.\textsuperscript{11} Co-localization studies revealed that hypercapnia significantly increased c-Fos expression in GABA-containing neurons in the medulla oblongata, especially in the ventral aspect of the medulla, within the Bötzinger region, the gigantocellular reticular nucleus, and the caudal raphe nuclei. An alternate approach, which we are pursuing, is the use of glutamic acid decarboxylase (GAD) isofrom staining as a marker for GABAergic neurons, as the GAD\textsubscript{65} and GAD\textsubscript{67} isoforms are largely responsible for GABA production.

From these combined physiologic and neuroanatomic data, we propose that GABAergic neurons in the medullary network modulate the ventilatory response to hypercapnia (Fig 2). Thus, CO\textsubscript{2} (potentially acting through interconnected glutamatergic neurons) excites respiratory output in parallel with GABAergic neurons which may inhibit the firing of cells that modulate inspiratory rhythm generation. These and future neuroanatomic observations, correlated with physiologic data, should allow better definition of the role that this and other specific neurotransmitter pathways play in modulating neonatal respiratory control.

\textbf{Role of GABA in Hypoxic Respiratory Depression}

Hypoxic exposure of many species in the newborn period results in a characteristic transient
increase and subsequent decrease in respiratory efforts, which is termed the biphasic ventilatory response. Although the response of the respiratory system to single episodes of hypoxia of varying length has been extensively investigated in humans as well as in anesthetized and unanesthetized animal species, studies have only recently begun to critically evaluate the effects of recurrent hypoxia on respiratory neural output. These studies are complicated by the fact that responses appear to differ widely, dependent on species studied, degree of maturation, confounding effects of anesthesia, and duration and magnitude of hypoxic exposures. We employed the anesthetized piglet model to begin to investigate the effects of recurrent hypoxia on respiratory neural output and whether GABA might be implicated in any cumulative inhibition of breathing induced by recurrent hypoxia. In response to multiple exposures to 8% oxygen (5-minute exposures, 10-minute recoveries) in 2- to 10-day-old animals, we observed that hypoxic depression of phrenic neural output progressively increased with subsequent exposures. This progressive increase in hypoxic respiratory depression could be largely reversed after intracisternal injection of the GABA\(_A\) receptor blocker bicuculline in the piglets (Fig 3). These data suggest that central GABAergic inhibition may contribute significantly to the cumulative inhibitory effects of repeated hypoxia in the newborn piglet model. It is unlikely that decreased peripheral chemoreceptor-mediated responses after recurrent hypoxic exposure contributed to these findings. Of interest in this regard are the recent data of Prabhakar in mature rats that recurrent episodes of short duration hypoxia actually increase carotid body firing.

**Figure 2.** Schematic model indicating the effect of increasing CO\(_2\) on the response of the neural network regulating inspiratory drive and respiratory timing. GLU, glutamate. Reprinted with permission.9

**Figure 3.** The increase in hypoxic ventilatory depression after recurrent hypoxia was reversed by administration of bicuculline in piglets ≤10 days of age (n = 7). Hypoxic exposure 1, ●—●; hypoxic exposure 6, □—□; hypoxic exposure after intracisternal bicuculline [40 µg/kg], △—△. Reprinted with permission.12
in response to subsequent hypoxic exposure,\textsuperscript{13} and the data of Nock and coworkers\textsuperscript{14} that preterm infants with more episodes of apnea actually exhibit greater increases in ventilation in response to hypoxic exposure.

Role of GABA in Apnea Induced by Laryngeal Stimulation

Stimulation of the laryngeal mucosa results in apnea in humans as well as in animals of different species. This reflex apnea is mediated via stimulation of the superior laryngeal nerve (SLN), and is most prominent during early maturation. It has been suggested that an exaggerated reflex response in newborns may be implicated in multiple pathological processes, including gastroesophageal reflux-induced apnea, sudden infant death syndrome, and apnea of prematurity. We hypothesized that upregulation of inhibitory neurotransmitter-mediated mechanisms in the immature brainstem might explain the vulnerability of newborns to this reflex, and again, that GABA might be implicated. In the unanesthetized 5- to 10-day-old piglet model, we performed graded SLN stimulation and measured phrenic neural output before and after GABA\textsubscript{A} receptor blockade with intravenous or intracisternal bicuculline. SLN stimulation caused a significant decrease in phrenic nerve amplitude, phrenic nerve frequency, minute phrenic activity, and inspiratory time that was proportional to the level of electrical stimulation.\textsuperscript{15} Increased levels of stimulation were more likely to induce apnea during stimulation that often persisted beyond cessation of the stimulus. Bicuculline, administered intravenously or intracisternally, decreased the SLN stimulation-induced decrease in phrenic nerve amplitude, minute phrenic activity, and phrenic nerve frequency (Fig 4). Bicuculline also reduced SLN-induced apnea and duration of poststimulation apnea. These findings are consistent with our observations during hypercapnic and recurrent hypoxic exposure, and point to a prominent role for GABA in the inhibitory timing mechanisms that are an integral component of neonatal respiratory reflex responses, and possibly apneic events.

Part Two

Adenosine in Neonatal Respiratory Control

A prominent role for adenosine in neonatal respiratory inhibition is suggested by the ability of the nonspecific adenosine receptor antagonists, theophylline and caffeine, to decrease the incidence of apnea of prematurity. Adenosine is a product of nucleotides such as ATP, and is formed as a consequence of metabolic and neural activity at many sites including the brain, especially during hypoxia. Although four major
adenosine receptor subtypes have been identified, the net effect of adenosine on neuronal activity depends primarily on a balance between activation of inhibitory A₁ and excitatory A₂ (especially A₂A) receptors. It has been widely presumed that the stimulatory effects of caffeine are secondary to A₁ receptor blockade, and this appears to be the case in lambs. Studies implicating adenosine in hypoxia-induced respiratory depression have employed primarily adenosine analogues and nonspecific adenosine antagonists. Meanwhile, molecular techniques have demonstrated widespread distribution of excitatory A₂A receptor mRNA in mature and developing rat brain. Of particular interest are the observations that adenosine A₂A receptors are expressed in GABA-containing striato-pallidal neurons and that GABA-release may be regulated via A₂A receptor activation. Based on our earlier findings of a prominent role for GABA in neonatal respiratory control, this led us to speculate that GABA release may be a mechanism underlying the respiratory inhibitory effects mediated by adenosine in early postnatal life.

**Role of GABA in Mediating Adenosine-Induced Respiratory Inhibition**

In a series of preliminary experiments in 5- to 10-day-old piglets, we sought to test the hypothesis that adenosine inhibits respiratory drive at this age via the selective activation of A₂A receptors. This, in turn, causes activation of GABAAergic inputs to the inspiratory drive center (pre-Bötzinger complex and associated inspiratory circuitry) located in the rostral ventrolateral medulla of the brainstem. In decerebrate animals (to avoid the effects of anesthesia), we measured phrenic neural output before and after intracisternal drug administration. Adenosine (5-50 μL of 10 or 50 mmol/L solution) invariably inhibited amplitude and frequency of phrenic neural output, and eventually resulted in apnea. Intracisternally administered bicuculline (50 μL) blocked this effect (Fig 5).

Injection of the selective adenosine A₂A receptor agonist (CGS21680, 50 μL) also resulted in brief apnea followed by respiratory inhibition. Intracisternal bicuculline injection (50 μL) again blocked the respiratory inhibitory effects of the adenosine A₂A agonist. These physiologic data, if supported by subsequent neuroanatomic data, strongly suggest that respiratory inhibition elicited by adenosine in this model may depend, in part, on GABAergic inputs to the brainstem inspiratory center.

**Conclusion and Clinical Application**

Xanthine therapy has been widely administered to diminish the incidence of apnea of prematurity for nearly 30 years. While these drugs are widely proven to increase central respiratory neural output to the respiratory muscles—probably via adenosine receptor antagonism—the precise mechanism whereby apnea is decreased by xanthines is unknown. In fact, the wisdom of such broad xanthine usage has been recently challenged, and an international multicenter randomized trial is currently testing the hypothesis that withholding such therapy may improve the neurodevelopmental outcome of preterm infants. Regardless of the outcome of that trial, it is important to clarify the mode of action of this therapy.

The data presented in this review provide evidence for a prominent role for GABA in inhibiting respiratory neural output in early development. Such evidence is best assembled by combining physiologic with neuroanatomic ex-
periments employing state-of-the-art experimental and molecular techniques. Perhaps more important, these data demonstrate the importance of understanding neurotransmitter interactions in elucidating the biologic basis of apnea in preterm infants. In this way, it may be possible to improve our pharmacologic interventions by developing specific, targeted approaches to the clinical problem.

References