Endogenous neuroprotection in the respiratory control system

By

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DEDICATIONS

This work is dedicated to my major professor, Dr. Stephen Johnson, for teaching me to go where the science leads, and also to my son and inspiration, Casyn Turner.

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ABSTRACT

Breathing must be robust and highly adaptable to maintain adequate oxygen and CO_2 levels during birth, development, pregnancy, and disease. This is achieved by a delicate balance of inhibitory and excitatory neuronal signaling. Both sustained and intermittent changes in respiratory neuron activity can create long-lasting changes in respiratory motor output *(i.e*., plasticity). As a constant requirement from birth until death, the respiratory control system must have endogenous mechanisms to maintain appropriate excitability during physiological or pathological stress, and express multiple types of plasticity. Reproduction is an example of an essential biological function with serious maternal and fetal risks. During late pregnancy, maternal brain allopregnanolone levels increase and augment the function of inhibitory $GABA_A$ receptors ($GABA_A$Rs), posing the risk of excessively inhibiting respiratory neurons. Here, we show that respiratory-related hypoglossal motoneurons increase epsilon subunit incorporation into $GABA_A$ receptors, which confers insensitivity to allopregnanolone. Similarly, brain allopregnanolone levels increase during the critical period in respiratory control development (occurs during the second postnatal week). We also found that epsilon subunit-containing $GABA_A$Rs dynamically change in respiratory-related brain regions during the second postnatal week. Thus, increased epsilon subunit incorporation in $GABA_A$Rs appears to protect breathing from excessive inhibition during pregnancy and postnatal development under physiological conditions. Thus, these studies suggest that adjusting $GABA_A$R subunit composition may be a little recognized, fundamental property of the respiratory control network. On the other hand, pregnancy and the neonatal period are also associated with pathological events, such as ischemic stroke. One potential strategy for protecting neurons from ischemia is to apply principles learned from ischemia-hypoxia resistant extremophile vertebrates, such as activating delta opioid receptors (DORs). We hypothesized that activating spinal DORs would prolong respiratory output (*i.e.*, provide neuroprotection) during oxygen-glucose deprivation (OGD; *in vitro* stroke model). We found that spinal DOR activation provides flexible neuroprotection against OGD,

regardless of whether DOR drugs are applied to the spinal cord before, during or after the onset of OGD. These studies suggest that understanding and controlling endogenous protective mechanisms is a compelling strategy for developing novel therapies and treatments to protect neuronal function against ischemia.

Chapter 1:

Introduction

Breathing must change rapidly in a controlled way to maintain adequate blood-gas homeostasis during birth, development, pregnancy, and disease. Thus, the respiratory control system is robust and highly adaptable, which is largely achieved through balancing GABAergic inhibition vs. glutamatergic excitation. As a constant requirement from birth until death, the respiratory control system must have endogenous mechanisms in place to maintain appropriate balance of inhibitory and excitatory signaling during common physiological stressors (*i.e.*, pregnancy, development) and also during pathological conditions, such as ischemia. This work focuses on two separate endogenous protective mechanisms to compensate for challenges in maintaining respiratory neuron function: 1) $GABA_AR$ subunit changes to protect again potential excessive neuronal inhibition caused by elevated brain neurosteroid levels and 2) delta opioid receptor (DOR) activation to protect neuronal function during excitotoxicity in an *in vitro* model of spinal neonatal stroke. Understanding and controlling endogenous protective mechanisms is a compelling strategy for developing novel therapies and treatments to protect neuronal function against physiological and pathological conditions.

Reproduction is an essential biological process during which hormonal changes can alter neuronal excitability. Thus, during pregnancy the respiratory control system naturally uses endogenous protective mechanisms to maintain appropriate neuronal activity. Central concentrations of the progesterone metabolite, allopregnanolone, increase 3-fold during pregnancy. Allopregnanolone is an inhibitory neurosteroid that enhances chloride ion influx through $GABA_ARs$ into neurons (*i.e.*, positive allosteric modulation) thereby increasing neuronal inhibition. Therefore, increased brain allopregnanolone levels increase the threat of respiratory depression. Breathing in pregnant animals is not inhibited, however, so, the respiratory control system must compensate for increased inhibitory signaling to maintain respiratory neuronal function. Shifting $GABA_AR$ subtypes is a potential mechanism to maintain neuronal excitation because the $GABA_AR$ subunit composition determines the receptor's electrophysiological properties and subcellular localization. Standard synaptic $GABA_ARs$ are

heteropentameric, and contain 2 alpha, 2 beta, and 1 gamma subunits, are sensitive to positive allosteric modulation, and widely expressed in respiratory-related neurons. When the relatively rare epsilon subunit is incorporated, $GABA_AR$s become resistant to positive allosteric modulation. *Our working hypothesis is that respiratory-related neurons increase epsilon subunit expression to protect breathing from excessive inhibition when brain allopregnanolone levels are increased.* We studied hypoglossal (XII) respiratory motoneurons because excessively inhibiting XII motoneurons may contribute to upper airway collapse, and potentially to sleep apnea pathogenesis. Our data suggest that XII motoneurons in pregnant rats increase epsilon subunit incorporation in $GABA_AR$s to become more resistant to allopregnanolone (Chapter 2). Increasing epsilon subunit expression in XII motoneurons to maintain upper airway patency could attenuate the sleep disordered breathing found in up to 40% of pregnant women. The mechanisms for maintaining respiratory motor network function during pregnancy may also apply to other examples of increased allopregnanolone concentrations, such as during development.

In young rats, there is a critical period in respiratory control development during the second postnatal week, mainly occurring from P11-P14. During the critical period, baseline respiratory frequency increases, the hypoxic ventilatory response decreases, neurotransmitter concentrations abruptly change, and receptor/subunit expression patterns shift towards the adult phenotype. Thus, while the critical period represents a time of rapid development, it is hypothesized to be a time of increased vulnerability in the respiratory control system. A key risk to breathing during this time is increased neuronal inhibition. The efficiency of neuronal inhibition increases due to increased GABA concentrations, as well as shifting $GABA_AR$ subunit composition and chloride transporters towards the adult phenotype. Another potential contributing factor to increased neuronal inhibition is increased central allopregnanolone levels at P10 and P14. Thus, we *hypothesize that epsilon subunit expression changes during the critical period to compensate for increased neuronal inhibition*. We found that

epsilon subunit incorporation into $GABA_A$Rs dynamically shifts during the critical period in medullary PBC-region, XII, and NTS neurons while non-respiratory cortical neurons remained relatively unchanged (Chapter 3). This is the first demonstration of a developmental role for epsilon subunit expression and suggests altered epsilon subunit expression may contribute to stabilizing respiratory neuron excitability. Our findings have potential clinical implications for pre-term infants (born at <34 gestational weeks) that have strikingly immature respiratory control systems and often suffer from central apneas (*i.e.*, apnea of prematurity). These infants also undergo a period of rapid respiratory control development until reaching full-term gestational age. Thus, understanding the endogenous mechanisms that create abrupt developmental changes and most importantly, stabilize breathing could have clinical implications for pre-term infants.

Finally, we studied an *in vitro* model of spinal excitotoxicity because the risk of ischemic stroke is significantly increased during the perinatal period. Perinatal ischemia is a significant clinical problem and can lead to lifelong deficits including motor disabilities and respiratory difficulties. Thus, it is crucial to protect spinal respiratory motoneurons from ischemia. While many neuroprotective agents have been studied, none have produced satisfactory results in clinical trials to date. The ideal neuroprotective agent against perinatal ischemia should be easy to administer, rapidly absorbed, and able to cross the placental and fetal blood-brain barriers with minimal or no adverse side effects. The ideal agent should activate endogenous neuroprotective mechanisms, disrupt the ischemic cascade at multiple points, and provide long-lasting (>24 h) protection no matter if given before, during, or after an unexpected perinatal ischemic event. Finally, ideal neuroprotective agents must preserve neuronal function rather than simply attenuating cell death. Therefore, in our studies we defined neuroprotection as the preservation of neuronal function, and quantified neuroprotection as continuation respiratory neuronal activity under conditions of increased GABAergic positive allosteric modulation or ischemia *in vitro*. DOR activation provides a unique form of neuroprotection from ischemic conditions that matches many

characteristics of the ideal neuroprotective agent because it appears to be a highly conserved, inducible mechanism, which is used by vertebrate extremophiles (*e.g.*, mammalian hibernators and hypoxia resistant vertebrates). Also, DOR-dependent protection is observed in various tissues other than brain, which makes it an attractive candidate for providing systemic protection during whole-body ischemia or hypoxia. DOR are expressed in neonatal animals, and DOR activation disrupts several steps in the ischemic cascade, provides long-lasting neuroprotection, and protects motor networks. Thus, we *hypothesize that spinal DOR activation will protect spinal respiratory motor networks from ischemia in vitro.* We showed that exogenous activation of this powerful endogenous mechanism significantly protects the respiratory motor network when DOR agonists are applied before, during or after the onset of ischemia (Chapter 4). Our data provide a compelling case for further study of DOR activation-dependent neuroprotection against neonatal stroke and for future studies of novel methods to induce DOR expression.

This work highlights the significant benefits of two separate endogenous neuroprotective mechanisms that protect breathing during challenges to neuronal excitability: 1) increased expression of the epsilon subunit in $GABA_A$Rs to confer resistance to positive allosteric modulation which protects neurons from excessive inhibition (Chapters 2, 3 and Appendix) and 2) activating DOR to disrupt multiple steps in the ischemic signaling cascade (Chapter 4).

I. Mammalian control of respiration

Within the respiratory system, lung/chest wall mechanics act as a pump to generate bulk gas flow (*i.e.*, convection) to ventilate the lungs. Then, respiratory gas exchange occurs across the alveolar-capillary membranes and the gases diffuse into the blood. Each step is required to maintain appropriate blood-gas homeostasis, and, if one function fails the system breaks down. Consequently, this process is a carefully coordinated unit that can meet changing requirements of gas exchange and is thus, under exquisite neuronal control. Respiratory-related neurons must generate the respiratory rhythm, transmit the signals to breath to motoneurons that innervate muscles to maintain airway patency and to pump air, and integrate chemo- and mechano- sensory inputs to create appropriate respiratory output. Since breathing continues throughout all phases of life and activity, the respiratory control system is both tightly regulated and highly adaptable. Neuronal respiratory control occurs through three major mechanisms: negative feedback (chemo- and mechanosensation), feedforward control (exercise hypernea; Forster *et al.*, 2012), and plasticity (Mitchell and Johnson 2003).

Plasticity in the respiratory control system

Neuroplasticity is a fundamental property of the respiratory control system that plays a critical role in adapting breathing to physiological changes (Mitchell and Johnson, 2003). Neuroplasticity can be defined as the ability of the nervous system to respond to intrinsic or extrinsic stimuli by reorganizing its structure, function, and connections (Cramer *et al.*, 2011). With respect to the respiratory control system, plasticity is often defined as a persistent change in the neural control system based on prior experience, and may involve structural or functional alterations (most commonly both), potentially arising from multiple cellular and synaptic mechanisms at different sites in the respiratory control system (reviewed in Mitchell and Johnson, 2003). Long-term functional and morphological changes underlying respiratory plasticity may be widely distributed throughout the respiratory control system

and can be induced by: hypoxia, hypercapnia, exercise, injury, stress, and pharmacological interventions or conditioning (Mitchell and Johnson, 2003). Another important feature of respiratory control plasticity is that it occurs during development (Ling *et al.*, 1997) as well as in adults (Kinkead *et al.*, 2001). Neuroplasticity can be manifested in a variety of ways, such as changes in: synaptic strength (Kempermann *et al.*, 2000; Kemp and Bashir, 2001), neuronal properties (Sperry and Goshgarian, 1993), neural network dynamics (Morris *et al.*, 2000, 2001, 2003), modulatory balance (Kinkead *et al.*, 2001) and the growth of new synapses (Liu and Chambers, 1958; Mamounas *et al.*, 2000).

With respect to our studies, a sustained increase in central allopregnanolone levels (increased neuronal inhibition) is sufficient to induce reconfiguration of $GABA_AR$ subunit composition in respiratory-related brain regions (Hengen *et al.*, 2012, Chapters 2, 3, and Appendix). We hypothesize this is a form of respiratory plasticity (based on the broader definition, Cramer *et al.*, 2011) because respiratory-related neurons change the subunit composition of $GABA_ARs$ to incorporate epsilon subunits. This changes the function of the receptor by conferring resistance to allopregnanolone, in order to maintain respiratory neuron excitability. This may be a novel form of respiratory plasticity that is critical to maintaining respiratory neuronal function during pregnancy (Hengen *et al.*, 2012; Chapter 2), development (Chapter 3), hypoxia (Johnson Lab, unpublished observations) or inflammation (Johnson Lab, unpublished observations). Finally, this work also demonstrates a separate example of respiratory plasticity induced by pre-treatment with a neuroprotective agent prior to the onset of ischemic conditions *in vitro*. Specifically, spinal delta-opioid receptor (DOR) activation for 10 min, followed by 20-min washout before spinal oxygen-glucose deprivation onset is sufficient to significantly prolong respiratory motor output compared to oxygen-glucose deprivation-only experiments (Chapter 4). This finding suggests that spinally activating DOR induces a long-lasting change that protects spinal respiratory motoneurons from ischemic conditions (*i.e.*, pharmacologically induced plasticity). Understanding respiratory plasticity may lead to insights into mechanisms that guide normal respiratory

control development and enable flexibility throughout life when confronted with changing circumstances (Mitchell and Johnson, 2003). Furthermore, studying respiratory plasticity in pathological states may provide the rationale for novel therapeutic intervention in clinical settings such as chronic lung disease, sudden infant death syndrome, sleep-disordered breathing, congenital alveolar hypoventilation syndrome, and neuromuscular injury (Mitchell and Johnson, 2003).

Key regions that need protection in the respiratory control system

Respiratory neurons must coordinate inspiration and expiration without pause and adapt, nearly instantaneously, to acute state changes (*e.g.*, sleep or exercise) and to long-term changes (*e.g.*, pregnancy, weight gain, or development) to ensure maintenance of blood gases and pH. Brainstem respiratory neurons oscillate in a 3-phase pattern: inspiration, post-inspiration (expiratory braking), and expiration (active expiration) (Richter and Spyer, 2001; Spyer and Gourine, 2009). There are several key components to respiratory control including: inspiratory respiratory rhythm (generated by Pre-Bötzinger Complex neurons, PBC), respiratory pattern formation (premotor neurons), motor output (via hypoglossal motoneurons [airway patency] and spinal motoneurons [pump air], chemosensitivity (raphe neurons, retrotrapezoid nucleus [RTN] neurons, parafacial respiratory group [FRG] neurons) and sensory integration of chemo- and mechanosensory inputs (nucleus tractus solitarius neurons [NTS]) chemo- and mechanosensory) (Fig. 1). Since all components are necessary to maintain proper respiratory control, successful neuroprotective strategies should be effective throughout respiratory-related brain regions. We studied a compensatory mechanism that protects neuronal function under conditions of increased neuronal inhibition in three key regions: PBC neurons, XII motoneurons, and NTS neurons (Chapter 2, 3, Appendix 1). In a separate project, we quantified neuroprotection from acute OGD-induced excitotoxicity by measuring spinal motoneuron respiratory output (Chapter 4).

Inspiratory rhythm generation: PBC neurons

The PBC is located bilaterally just ventral to the subcompact nucleus ambiguus and is composed of about ~1,000 neurons per side that co-express neurokinin-1 (NK1) receptors, somatostatin 2A receptors and the glycoprotein reelin (Tan *et al.*, 2012; Gray *et al.*, 2010, Feldman *et al.*, 2013). However, the PBC is best defined functionally, rather than anatomically. Known as the principal kernel or "noeud vital" of respiratory rhythm generation, the PBC contains glutamatergic interneurons whose circuitry produces basic inspiratory activity, in which pacemaker neurons can be embedded but are not required for rhythm generation (Funk *et al.*, 1993; Wallen-Mackenzie *et al.*, 2006; Feldman *et al.*, 2003; Feldman and Del Negro, 2006; Smith *et al.*, 2013). Mechanisms underlying inspiratory rhythm generation are a source of controversy in the literature (reviewed in Feldman *et al.*, 2013). One current hypothesis is that small clusters of neurons generate rhythmic activity that is related to spontaneous, metabotropic glutamate-dependent calcium waves that are initiated in dendrites and propagate towards the soma (Mironov, 2008). These calcium waves may be generated by PBC interneurons mutually interacting to increase postsynaptic intracellular calcium levels via metabotropic glutamate receptor (mGluR) activation and AMPA receptor-mediated recruitment of calcium channels causing the typically latent calcium-activated non-specific cation currents (I_{CAN}) to be evoked synaptically (Del Negro *et al.*, 2011; Del Negro and Hayes, 2008), as well as activation of transient receptor potential channels (TRPM4/5) at the start of inspiration (Mironov, 2008; Pace *et al.*, 2007; Crowder *et al.*, 2007; Del Negro *et al.*, 2010).

As the inspiratory rhythm generator, PBC neurons are highly interconnected with many synaptic projections and electrical synapses (Rekling *et al.*, 2000; Hayes and Del Negro, 2007). Additionally, each PBC neuron sends an axon towards the midline, presumably towards the contralateral PBC (Feldman *et al.*, 2013). The PBC receives tonic drives from the pons and multiple medullary chemoreceptive sites (Rybak *et al.*, 2007). NTS neurons (discussed below) provide excitatory input to PBC rhythm generating circuitry (Guyenet 2008). With respect to output, major projections of the PBC include premotor

neurons in the rostral ventral respiratory group and also the hypoglossal nucleus (Tan *et al.*, 2010). The output of the PBC is necessary and sufficient for the generation of hypoglossal nerve motor output *in vitro* (Feldman *et al.*, 1990; Smith *et al.*, 1991).

We recorded from PBC neurons because protecting the inspiratory rhythm generator is critical for sustaining life during challenges to neuronal excitability. Therefore, we hypothesize PBC may be more likely than other respiratory-related nuclei to utilize endogenous protective mechanisms to ensure maintenance of rhythmogenesis. Data in Chapter 3 and Appendix support this hypothesis.

Upper airway patency: XII neurons

The hypoglossal (XII) motor nucleus is mainly composed of two types of neurons: motoneurons and interneurons. XII motoneurons are large (25-50 μm), multipolar in shape, and widely distributed with a large, round, centrally located nucleus (Boone and Aldes, 1984). XII motoneurons receive inputs from the cortex, medulla and pons (Peever *et al.*, 2002). Synaptic inputs from central pattern generators govern XII motoneurons activity for a variety of behaviors including breathing, chewing, suckling, and swallowing (Lowe 1980, Ono *et al.*, 1998; Wrobel *et al.*, 2010). The hypoglossal nucleus may contain distinct functional networks such that area of the dorsal medulla in and around the solitary tract contains neurons with inputs to tongue retractor motoneurons and the region ventrolateral to the hypoglossal nucleus contains circuitry specific to tongue protruder motoneurons (Dobbins and Feldman, 1995). Much like other motoneurons, XII motoneurons have a variety of membrane receptors including ionotropic receptors involved in fast excitatory/inhibitory synaptic transmission: glutamatergic, GABAergic, and glycinergic receptors as well as metabotropic receptors responsible for cholinergic, adrenergic, glutamatergic and peptidergic neurotransmission and modulation (reviewed in: Rekling *et al.*, 2000). Respiratory drive to XII motoneurons comes from PBC neurons (Tan *et al.*, 2010), premotor neurons scattered throughout the lateral tegmental field (Peever *et al.*, 2002), and interneurons within

the XII motor nucleus (Peever *et al.*, 2002). The nucleus of Roller sends inhibitory inputs to the XII (van Brederode *et al.*, 2011) XII motoneurons are postsynaptically excited during inspiration (Remmers *et al.*, 1978; Fregosi and Fuller, 1997) causing activation of the XII nerve which innervates extrinsic and intrinsic tongue muscles, thereby contributing to maintaining pharyngeal airway patency through decreasing airway resistance (Remmers *et al.*, 1978; Fregosi and Fuller, 1997; Wrobel *et al.*, 2010). During inspiration, XII motoneurons membrane potentials are depolarized at a maximum discharge rate of 90 Hz while receiving concurrent glutamatergic excitation and GABAergic inhibition (Rekling and Laursen, 1989; Viana *et al.*, 1990).

In contrast, interneurons make up only ~11% of all neurons within the XII motor nucleus in mice (Sturrock *et al.*, 1991). XII interneurons are small (10-18 μm), round to oval shaped and located on the ventral and dorsolateral edges of XII with limited dendritic aborizations (Peever *et al.*, 2002). XII interneurons have distinct electrophysiological characteristics including: long latency synaptic potentials following XII nerve stimulation (Green and Negishi, 1963; Sumi, 1969; Takata, 1993) and a maximum discharge rate of 250 Hz (Rekling and Laursen, 1989; Viana *et al.*, 1990; Peever *et al.*, 2002). Also, XII interneurons appear to receive excitation from respiratory neurons and putatively contact XII motoneurons dendrites at GABAergic terminals resulting in concurrent excitation and inhibition of XII motoneurons during inspiration (Takata 1993, Withington-Wray *et al.*, 1988; Woch and Kubin 1995; Peever *et al.*, 2002).

We studied the XII motor nucleus (Chapters 2 & 3, Appendix) because XII motoneurons play an important role in the control of breathing. Enhanced inhibitory signaling in XII motoneurons could lead to upper-airway collapse and may contribute to sleep apnea pathogenesis. To adequately protect blood-gas homeostasis all major contributors to respiratory control must have endogenous protective mechanisms, however, specific mechanisms may differ between chemosensitive sensory relay neurons, inspiratory rhythm generating neurons, and motoneurons as shown in Chapter 3.

Sensory integration: NTS neurons

The NTS is located in the dorsomedial medulla (Spyer and Gourine, 2009), contains CO_2-sensitive cells (Coates *et al.*, 1993; Dean *et al.*, 1989), and receives the first synapse from O_2-sensitive sensory afferents from the carotid bodies (Housely *et al.*, 1987; Housely and Sinclair, 1988; Vardhan *et al.*, 1993). The NTS receives the majority of cardiovascular and respiratory afferent information from the vagal and glossopharyngeal nerves (Spyer and Gourine, 2009). In fact, sensory systems that synapse in the NTS include (but not only) peripheral chemoreceptors, baroreceptors, and mechanoreceptors (Donoghue *et al.*, 1984; Jordan and Spyer, 1986). Chemoreceptor and baroreceptor afferents are synaptically linked to different populations of NTS neurons although they do converge onto ~40% of NTS cells (Mifflin 1992, 1993; Paton, 1998, Silva-Carvalho *et al.*, 1998) and these neurons likely contribute to cardiac vagal activity (Paton *et al.*, 2001). Thus, NTS neurons are heterogeneous mix of primary sensory relay neurons and chemo- and mechanosensitive neurons. Consequently, the NTS contains several subnuclei including the medial, dorsomedial, and commissural subnuclei which are strongly innervated by chemoreceptor afferents (Paton *et al.*, 2001). Second order, chemoreceptor NTS neurons are mainly located in the commissural subnucleus (Paton *et al.*, 2001). In contrast, respiratory neurons are located in the ventrolateral subnucleus of the NTS, a region that also has significant innervations by chemoreceptor afferents (Paton *et al.*, 2001). As the NTS receives inputs from a wide variety of sources, the nucleus is not coded by the nature of the inputs but rather by the efferent connections of the nucleus (Spyer and Gourine, 2009). The main respiratory-related outputs from the NTS are powerful excitatory inputs to the RTN/pFRG and inspiratory rhythm generating circuitry in the PBC (Guyenet 2008).

We recorded from NTS neurons because maintaining appropriate function in chemosensitive neurons is critical for blood-gas homeostasis. With respect to respiratory-related function, enhanced inhibitory signaling in chemo-sensitive NTS neurons could decrease respiratory drive by decreasing the

excitatory drive NTS neurons provide to inspiratory rhythm generating PBC neurons. Our recordings were focused in the caudal NTS (ventral to the area postrema) because that is the area in the rat NTS where the majority of CO_2-sensitive neurons are located under *in vitro* conditions (Dean *et al.*, 1989; 1990) and also contains secondary neurons receiving afferent input from the arterial chemoreceptors that respond to O_2 and CO_2 (Donoghue *et al.*, 1984; Housley *et al.*, 1987, 1988). Thus, the majority of our data are from the commissural and dorsal medial subnuclei of the NTS and likely representative of chemosensitive NTS neurons (Chapter 3, Appendix 1).

Diaphragm and Intercostals muscles innervation: spinal motoneurons

Respiratory muscles of the upper airways, diaphragm, and intercostal muscles receive projections from at least two general descending pathways: the bulbospinal and corticospinal pathways. Bulbospinal medullary projections are responsible for the automatic control of breathing whereas corticospinal projections from the primary motor cortex are responsible for conscious/ voluntary control of breathing (reviewed in Butler, 2007). The rat phrenic motor nucleus, which is located at cervical cord levels C_3-C_5, innervates the diaphragm. Further caudal in the cord, intercostal motoneurons located at thoracic cord levels T_5-T_7 innervate the intercostal muscles of the rib cage. We used the brainstem-spinal cord preparation (reviewed in Johnson *et al.*, 2012) to record respiratory motor output from phrenic and intercostals nerve roots in neonatal rats. This preparation keeps a large portion of the respiratory network intact including the PBC, which excites premotor neurons densely located in the caudal ventral respiratory group (Tian and Duffin 1996; Peever *et al.*, 2001). Premotor neurons relay respiratory signals to the spinal motoneurons which are longitudinally organized in the ventral horn of the spinal cord (Berger, 2011). Spinal motoneurons and XII motoneurons have distinct premotor signaling pathways (Peever *et al.*, 2001) in which there is little cross-talk such that only ~4% of phrenic premotor neurons also excite XII motoneurons (Peever *et al.*, 2001, 2002). We recorded spinal respiratory motor output in

brainstem-spinal cord preparations to quantify motor system function, compare spinal function between motor pools, and to study neuroprotection specifically within spinal motor circuits.

III. $GABA_AR$ subunit composition and function

Basic characteristics of $GABA_ARs$

While this work focuses only on the $GABA_AR$, there are three subtypes of receptors that bind the neurotransmitter GABA: $GABA_A$ receptors, $GABA_B$ receptors, and $GABA_\rho$ receptors (Nakayasu *et al.*, 1995; Bormann, 2000). $GABA_B$ receptors are less common than $GABA_A$ receptors, are metabotropic, and therefore operate via a G-protein linked cascade to open potassium channels, to cause hyperpolarization (Crunelli and Leresche, 1991). $GABA_\rho$ (previously $GABA_C$) receptors are a specific subtype of $GABA_A$ receptor composed of five ρ subunits (Zhang *et al.*, 2001).

$GABA_AR$ are ligand-gated ion channels in the cys loop superfamily and are widely accepted as heteropentameric receptors (Bonnert *et al.*, 1999). GABA binding to the pentamer opens the anion-selective intrinsic channel through which primarily chloride ions flow resulting in fast synaptic transmission. The influx of chloride ions brings the cell's resting potential closer to the chloride reversal potential (Katzung, 2001), which is usually hyperpolarizing (the neonatal period is one exception). Therefore, GABA-mediated currents are inhibitory and decrease the likelihood that the postsynaptic neuron will fire an action potential. $GABA_ARs$ have a wide range of specific properties due to subunit composition and GABA handling in and around the synaptic cleft which varies greatly (for review see Mody and Pearce, 2004). Subunit composition determines the receptor's electrophysiological properties and subcellular localization. In total, $GABA_AR$ are composed of 18 different subunits divided into eight

subunit families based on amino acid sequence homology: alpha-1-6; beta-1-3, gamma-1-3, delta, epsilon, pi, theta-1-3, and rho-1-3 (Sieghart and Sperk, 2002; Glykys *et al.*, 2007).

In common synaptic $GABA_A$Rs, the five subunits that form the chloride pore are two alpha, two beta, and one gamma subunits (Sieghart and Sperk, 2002). Following action potential-mediated presynaptic vesicular release of GABA (low mM concentrations) standard $GABA_A$Rs are activated by two molecules of GABA binding to the two alpha-beta interfaces, which opens the chloride ion pore and creates a phasic GABA-dependent inhibitory postsynaptic current (IPSC). The gamma subunit interacts with the anchoring protein (gephyrin) and is essential for postsynaptic clustering (Essrich *et al.*, 1998; Alldred *et al.*, 2005). While there are many possible combinations of five subunits, only a few dozen are thought to exist in the mammalian brain due to specific subunit partnerships (Sieghart and Sperk, 2002; Glykys *et al.*, 2007). Incorporating non-standard $GABA_A$R subunits into the pentamer confers distinct characteristics to $GABA_A$Rs. For example, $GABA_A$Rs that contain epsilon or delta subunits may not contain a gamma subunit or contribute to phasic IPSCs (Brickley *et al.*, 1996). $GABA_A$R that lack a gamma subunit can be found in perisynaptic or extrasynaptic locations rather than localized to a postsynaptic density (Essrich *et al.*, 1998; Alldred *et al.*, 2005). Extrasynaptic $GABA_A$Rs, activated by ambient, nanomolar concentrations of GABA that fluctuate minimally (Mody and Pearce, 2004; Farrant and Nusser, 2005; Wagner *et al.*, 2005), and mediate a tonic inhibitory current. This work focuses on mainly on epsilon subunits and also delta subunits because of the unique sensitivity to positive allosteric modulators incorporation of these subunits confer to the receptor.

Positive allosteric modulation in $GABA_A$Rs

While GABA directly activates $GABA_A$Rs, positive allosteric modulators potentiate $GABA_A$R activity by increasing channel open time, thereby allowing enhanced chloride ion influx in both synaptic

and extrasynaptic $GABA_A$Rs. Examples of positive allosteric modulators include: barbiturates (*e.g.*, pentobarbital; Sancar and Czajkowsi, 2010), neurosteroids (*e.g.*, allopregnanolone; Baker *et al.*, 2010), benzodiazepines (Orser, 2006), and ethanol (Mody *et al.*, 2007). Positive allosteric modulators bind to sites distinct from GABA. For example, neurosteroids bind to a cavity formed by the alpha-subunit transmembrane domain (M1-M4 regions; Hosie *et al.*, 2006). The neurosteroid, allopregnanolone, is a progesterone metabolite and is also synthesized in the brain by neurons and glia (Zheng, 2009). Additionally, allopregnanolone alters release of several neurotransmitters (*e.g.*, serotonin, norepinephrine, and dopamine) in different parts of the brain and directly inhibits L-type calcium channels in the prefrontal cortex (Hu *et al.*, 2002; Zheng, 2009).

$GABA_A$R subunit composition regulates regional responses to positive allosteric modulators. A compelling protective strategy to protect neurons from excitability dysregulation is changing the subunit composition of $GABA_A$R to confer desirable properties to the neuron. For example, extrasynaptic delta subunit-containing $GABA_A$Rs are hypersensitive to positive allosteric modulation (Olsen *et al.*, 2007; Hevers and Lüddens, 1998) while epsilon-containing $GABA_A$Rs are resistant (Irnaten *et al.*, 2002; Wagner *et al.*, 2005). Thus, increasing epsilon subunit incorporation in $GABA_A$Rs will protect neurons from excessive allopregnanolone-dependent inhibition. Inclusion of epsilon or delta subunits in $GABA_A$Rs confers distinct properties to neurons which are discussed in detail below.

Unique characteristics of $GABA_A$Rs incorporating epsilon subunits

Epsilon expression patterns: Relatively little is known regarding the physiological role of epsilon subunits in native receptors, although $GABA_A$Rs containing epsilon subunits are found in cholinergic, dopaminergic, serotonergic, and noradrenergic neurons that have neuromodulatory actions (Belujon *et al.*, 2009). Epsilon mRNA expression is restricted to hypothalamus, hippocampus, medulla and spinal

cord in monkeys (Whiting *et al.*, 1997), however, epsilon subunit mRNA is expressed throughout the rat brainstem including the raphe nuclei, A5 area, NTS, locus coeruleus, and dorsal vagal complex (Moragues *et al.*, 2000; Kasparov *et al.*, 2001). Epsilon subunit expression may also have a developmental role as epsilon mRNA expression steadily increases from embryonic day 14 (E14) to postnatal day 12 (P12) in rat medulla (Pape *et al.*, 2009). Taken together these studies suggest that epsilon subunits may have a widespread and currently little appreciated role in modulating neuronal activity.

Electrophysiological properties in recombinant receptors: The epsilon subunit was first cloned in 1997 (reported simultaneously by Davies *et al.*, and Whiting *et al.*) and many studies since have described unique and paradoxical biophysical and pharmacological properties in recombinant receptors containing alpha-beta-epsilon subunits (Jones and Henderson, 2007). Recombinant receptors that contain epsilon subunits are gated by GABA (Neelands *et al.*, 1999; Davies *et al.*, 2001; Maksay *et al.*, 2003; Wagner *et al.*, 2005; Jones *et al.*, 2006). However, inclusion of the epsilon subunit confers the following properties to $GABA_A$Rs: spontaneous opening, insensitivity to benzodiazepines, slow receptor deactivation, altered receptor desensitization, altered sensitivity to positive modulation by anesthetics and neurosteroids and negative modulation by anabolic androgenic steroids (Davies *et al.*, 1997, 2001; Whiting *et al.*, 1997; Thompson *et al.*, 1998, 2002; Neelands *et al.*, 1999; Maksay *et al.*, 2003; Wagner *et al.*, 2005; Jones *et al.*, 2006). Published data are conflicting on the ability of anesthetics and anesthetic neurosteroids to potentiate epsilon-containing recombinant $GABA_A$Rs (Davies *et al.*, 1997, 2001; Whiting *et al.*, 1997; Thompson *et al.*, 1998). This may be due to the relative concentrations of epsilon subunit vs. alpha and beta subunits and that high ratios of epsilon (1:1:1) in the constructs produce resistance to pentobarbital and allopregnanolone, but, lower ratios do not (1:1:0.1-0.01 = sensitive to pentobarbital; Thompson *et al.*, 2002). Interestingly, at concentrations sufficient to increase resistance to positive

allosteric modulators there is also a significant increase in spontaneous channel openings. However, the spontaneous activity varies widely depending on construct identity and transfection conditions in recombinant receptors. Thus, there appears to be an expression threshold at which positive allosteric modulation resistance and tonic current properties are reached. This data may also suggest that spontaneous GABA-independent tonic current and resistance to positive allosteric modulation are physiological features of epsilon subunits that are co-expressed in $GABA_AR$.

Epsilon subunit assembly patterns in $GABA_ARs$: The epsilon subunit must combine with at least one alpha subunit and one beta subunit to form a functional receptor with GABA-activated currents and ligand binding (Davies *et al.*, 1997; Whiting *et al.*, 1997). When epsilon is expressed alone or in combination with either only alpha or only beta subunits there is no significant ligand-binding or ligand-induced channel activity (Davies *et al.*, 1997; Whiting *et al.*, 1997). In addition, epsilon and gamma subunits share the greatest amino acid sequence homology (38-47%; Bollan *et al.*, 2008); thus, it was widely thought that epsilon subunits substituted for gamma subunits. Supportively, in instances when epsilon expression is high, gamma subunit expression is low. For example, in the locus coeruleus in situ hybridization shows significant epsilon expression while gamma-1-3 subunits are undetectable (Belujon *et al.*, 2009). However, various alternative assemblies for $GABA_AR$ containing epsilon subunits were recently proposed (Jones *et al.*, 2007; Bollan *et al.*, 2008). Alternative assemblies could include multiple epsilon subunits within a receptor or different positioning of a single epsilon subunit within the pentamer (Jones *et al.*, 2007). Various combinations of subunits in epsilon-containing $GABA_ARs$ may also explain pharmacology discrepancies in these receptors and contribute to functional variability by promoting altered subunit interactions or distinct post-translational modifications (Jones *et al.*, 2007). Incorporating more than one epsilon subunit into a pentamer could alter the allosteric modulation properties (Olsen, 1998), channel gating (Gingrich *et al.*, 1995; Lavoie *et al.*, 1997), formation of GABA

binding sites (Kash *et al.*, 2003) and current rectification (Davies *et al.*, 1997, 2001). Based on studies that tested various subunit combinations in recombinant receptors and measured spontaneous currents, evoked currents and surface receptor expression; the epsilon subunit is most likely to replace alpha-1 at position 1, beta-2 at position 4 or gamma-2 at position 5 (Bollan *et al.*, 2008). While studies on recombinant receptors provide critical information on subunit-physiology, it remains unknown which assemblies are present in native receptors.

Epsilon subunit expression in native $GABA_A$Rs: In our models of increased natural epsilon subunit expression, epsilon subunits appear to confer resistance to positive allosteric modulators *in vitro* including allopregnanolone (Chapter 2), pentobarbital (Chapters 2, 3; Appendix; Hengen *et al.*, 2009, 2011, 2012) and ethanol (Hengen *et al.*, 2011). Further, elevated central allopregnanolone levels appear to be capable of increasing epsilon subunit incorporation (Chapter 2, Appendix, Hengen *et al.*, 2012). It remains unclear whether native neurons thought to express epsilon have a spontaneous, GABA-independent current (Kasparov *et al.*, 2001; Irnaten *et al.*, 2002; Jorge *et al.*, 2002; Sergeeva *et al.*, 2005; Jones *et al.*, 2006). Thus, the work from our group suggests that the key physiological role of natural epsilon expression may be to provide protection from enhanced inhibition due to increased central allopregnanolone concentrations (Hengen *et al.*, 2009, 2011, 2012; Chapters 2, 3, Appendix). This endogenous protective mechanism appears to be utilized specifically by respiratory-related neurons as cortical neurons did not increase epsilon expression in any of the physiological models studied: hibernation (Hengen *et al.*, 2009, 2010), pregnancy (Hengen *et al.*, 2012) and the critical period in respiratory control development (Chapter 3).

Unique characteristics incorporating the delta subunit into $GABA_A$Rs confers to neurons:

Delta subunit expression patterns: Delta subunits confer distinct properties to $GABA_A$Rs that sharply contrast characteristics attributed to the inclusion of epsilon subunits. Delta subunits are expressed in the cortex, hippocampus, dentate gyrus, cerebellum, thalamus, and striatum (Sur *et al.*, 1999; Pirker *et al.*, 2000; Sun *et al.*, 2004; Peng *et al.*, 2002; Maguire *et al.*, 2009). With respect to the medulla, delta subunits are expressed in rat medial NTS neurons (Herman *et al.*, 2012); in XII motoneurons from P3-P15 rats (Numata *et al.*, 2012); and also in the rostral ventrolateral medulla in adult hibernating ground squirrels (Hengen *et al.*, 2011). However, the role of delta subunit containing $GABA_A$R in respiratory control is still unclear.

Electrophysiological properties delta subunits confer to $GABA_A$Rs: The main physiological role of $GABA_A$R containing delta subunits is to mediate a GABA-dependent tonic current. $GABA_A$Rs containing delta subunits cannot accumulate at postsynaptic sites, likely because they cannot anchor to the postsynaptic scaffold protein complex (Farrant and Nusser, 2005). Thus, $GABA_A$R containing delta subunits are found in peri- or extrasynaptic locations and sense GABA following vesicular release from nearby boutons or from ambient GABA in the extracellular space (Wei *et al.*, 2003; Glykys and Mody, 2006). Receptors containing the delta subunit have orders of magnitude higher affinity for GABA (Wallner *et al.*, 2002) and desensitize more slowly than standard $GABA_A$RS (Storustovu and Ebert, 2006) or do not show desensitization (Wallner *et al.*, 2002). Despite high GABA binding affinity, GABA has low efficacy in channel gating (poor agonist) suggesting that enhancement of GABA efficacy through channel modulation (*i.e.*, allopregnanolone or pentobarbital) is the main mechanism for activating these receptors (Glykys and Mody, 2006). Delta subunit containing $GABA_A$R are modulated by physiological concentrations of neurosteroids and by low concentrations of ethanol (Wei *et al.*, 2004; Stell *et al.*, 2003; Hancher *et al.*, 2005). Thus, delta-containing $GABA_A$R are extremely sensitive to potentiation by

positive allosteric modulators including potentiation by neurosteroids (Wohlfarth *et al.*, 2002). Supportively, mice lacking the delta subunit are markedly less sensitive to neurosteroids in vivo (Mihalek *et al.*, 1999) and in vitro (Spigelman *et al.*, 2003). Therefore, delta subunit containing GABAAR have distinct electrophysiological properties.

Subunit assembly patterns in delta-containing $GABA_AR$: Delta-containing $GABA_A$Rs generally co-assemble with alpha-4 or alpha-6 subunits (Sur *et al.*, 1999; Jones *et al.*, 1997) and lack a gamma subunit (Araujo *et al.*, 1998; Quirk *et al.*, 1995). These assembly patterns seem to follow specific partnership rules as cerebellar granule cells that lack alpha-6 subunits also lack delta subunits (Jones *et al.*, 1997). Further, $GABA_A$R that contain gamma subunits are anchored at the synapse while delta-containing $GABA_A$Rs are located extra- or perisynaptically (Sun *et al.*, 2004; Nusser *et al.*, 1998; Wei *et al.*, 2003), suggesting little overlap between subunit pairs. However, a surprising natural partnership between alpha-1 and delta subunits occurs in the dentate gyrus molecular layer that are extremely sensitive to ethanol (Glykys *et al.*, 2007). This illustrates that unexpected combinations of $GABA_A$Rs subunits exist and confer unique properties to $GABA_A$Rs in the mammalian brain and also that the stoichiometry of subunits in native receptors cannot be assumed based on general expression patterns.

Role of delta subunits when brain allopregnanolone levels are increased: Based on this work, we hypothesize that delta subunits may decrease when expression of the epsilon subunit increases in respiratory-related neurons. We found that delta subunit mRNA decreases in the cortex, XII and PBC-region during pregnancy (Chapter 2) and our electrophysiological data suggests a decrease in delta subunits in XII and PBC-region neurons following daily treatment with a progesterone analogue to pharmacologically mimic pregnancy in young rats (Appendix).

III. $GABA_A$Rs in respiratory control

$GABA_A$Rs are required for proper function in respiratory-related neurons

$GABA_A$R function is required for proper respiratory rhythm generation: Signaling through $GABA_A$Rs is required within the respiratory control system and maintaining appropriate GABAergic tone is critical for respiratory neuron function. For example, enhancing GABA with the agonist, muscimol (microinjected bilaterally into the caudal ventrolateral medulla), blocks the sympathetic baroreflex (inhibits neuronal firing in the NTS), rapidly eliminates respiratory motor outflow from the XII nerve (inhibits neuronal firing in PBC and XII neurons), and disrupts reciprocal synaptic inhibition in rostral ventrolateral medullary neurons (Koshiya and Guyenet, 1996). In contrast, $GABA_A$R blockade (bicuculline, 1-50 μM) also significantly disrupts of XII motor output, causing sustained depolarization and oscillations in the cell membrane potential (Paton and Richter, 1995). Thus, excessive or insufficient GABAergic signaling within the respiratory control system significantly disrupts neuronal function.

Role of synaptic inhibition in respiratory rhythm generation: While inhibitory signaling is clearly important for respiratory control, new controversy has emerged regarding the role of inhibitory signaling in respiratory rhythm generation. A well-known hypothesis is that rhythmic movements in mammals (*e.g.*, breathing, suckling, walking) are generated by mutually inhibitory groups of neurons (Brown, 1914). This idea was first applied to the respiratory control system in 1963 (Burns) and has been refined through many subsequent studies. Respiratory rhythm generation is generally thought to require synaptic inhibition (GABAergic and glycinergic) between two or three groups of neurons (Burns, 1963; Richter, 1982; Richter *et al.*, 1992; Smith *et al.*, 2007, 2013). In the current model, inhibitory expiratory neurons in the Bötzinger complex and inhibitory inspiratory neurons in the PBC are coupled

in a ring-like network with mutual inhibitory interactions (Smith *et al.*, 2007, 2013; Rybak *et al.*, 2009; Rubin *et al.*, 2009). The hypothesis predicts that the inhibitory network interacts with excitatory PBC neurons to coordinate inspiratory and expiratory phases (Smith *et al.*, 2007). Also, the inhibitory network receives drives from pontine, RTN/pFRG, and raphe neurons to modulate output from the PBC-Böztinger complex microcircuit (Smith *et al.*, 2013). Thus, in this model, postsynaptic inhibition is crucial for inspiratory and expiratory rhythm generation.

However, a new study suggests the primary role of inhibitory signaling within the respiratory control system is to shape the pattern of respiratory motor output, stabilize breathing, and to mediate reflex or volitional apnea, rather than to generate the rhythm itself (Janczewski *et al.*, 2013). In this study, GABAergic and glycinergic inhibition were blocked with specific antagonists microinjected into the PBC and Bötzinger complex of anesthetized, spontaneously breathing adult rats (Janczewski *et al.*, 2013). GABA and glycine blockade was sufficient to suppress the Breuer-Herring inspiratory inhibitory inflation reflex, but a normal breathing rhythm continued (Janczewski *et al.*, 2013), thereby suggesting postsynaptic inhibition within the PBC or Bötzinger complex is not required for rhythm generation. Previous studies in reduced preparations from immature rats also suggest inhibitory signaling is not required for rhythm generation. For example, in arterially perfused *in situ* brainstem-spinal cord preparations, GABA and glycine receptor blockade changes respiratory burst shape and regularity although respiratory rhythm continues (St-John *et al.*, 2009). Similarly, in brainstem-spinal cord and medullary slice preparations *in vitro*, respiratory motor output continues during bath application of GABA and glycine receptor antagonists (Feldman and Smith, 1989; Shao and Feldman, 1997). Taken together, these studies suggest a role for GABAergic signaling in respiratory control beyond basic synaptic inhibition.

Role of gain modulation in respiratory control: Another role for GABAergic signaling in the respiratory control system is gain modulation. Gain is the ability of an amplifier to adjust the output of a signal relative to an input. Gain modulation stabilizes neural network activity during plasticity (Turrigiano and Nelson, 2004) and allows a system to express nonlinear, dynamic control of output relative to input. The respiratory control system utilizes gain modulation to constrain neuronal firing rates by 50-65% in premotor neurons located in the ventral respiratory group (Zuperku and McCrimmon, 2002) and by 25% in XII motoneurons (Sanchez *et al.*, 2008), under baseline conditions. Relieving this constraint by blocking $GABA_A$Rs results in an amplified replica of baseline respiratory output, thereby demonstrating that a tonic GABAergic inhibitory input constrains neuronal firing rates (Zuperku and McCrimmon, 2002). Further, GABAergic gain modulation is multiplicative rather than linear because there is not an increased y-intercept with a constant slope (a hallmark of an additive process; Zuperku and McCrimmon, 2002) which suggests a well-controlled broad range of flexible neuronal activity. Finally, the gain modulatory effect of $GABA_A$R blockade is likely due to a reduction of GABAergic shunting inhibition (Tonkovic-Capin *et al.*, 2001). Gain modulation in XII motoneurons is bicuculline ($GABA_A$R antagonist)-sensitive and picrotoxin (chloride channel antagonist)–insensitive suggesting incorporation of subunits other than those in standard $GABA_A$Rs (Sanchez *et al.*, 2008). Nonetheless, the subunit composition of $GABA_A$Rs responsible for gain modulation is not known. Such gain control could provide a powerful mechanism for the respiratory control system to optimize breathing pattern in response to changes in condition or state (McCrimmon *et al.*, 1997).

Role of epsilon subunit containing $GABA_A$R in respiratory control: The potential role for epsilon subunit expression in the respiratory control system has only recently been discovered (Hengen *et al.*, 2009). In these studies, epsilon subunit expression increased during hibernation in ventral respiratory group neurons compared to summer active squirrels suggesting a novel mechanism to maintain

breathing when neuronal activity in other brain regions is significantly attenuated (Hengen *et al.*, 2009, 2011). It was hypothesized that an endogenous substance increased centrally (*e.g.*, allopregnanolone) and was sufficient to inhibit cortical but not medullary neurons, which contribute to maintenance of cardiorespiratory function during hibernation. One potential mechanism for maintaining respiratory control is increased epsilon subunit incorporation into $GABA_AR$s to confer resistance to endogenous positive allosteric modulators of GABAergic inhibition. The findings that brainstem neurons increase epsilon subunits during hibernation (Hengen *et al.*, 2009, 2011) and pregnancy (Hengen *et al.*, 2012, Chapter 2), but, cortical neurons do not (Hengen *et al.*, 2009, 2010, 2012, Chapter 3), suggest epsilon subunits play a key role in protecting and modulating cardiorespiratory function.

We hypothesize that epsilon subunit expression increases to confer resistance to positive allosteric modulation in $GABA_AR$. However, other properties associated with epsilon subunit expression have not been studied in native, respiratory neurons. One potential trade-off to increased epsilon subunit incorporation into $GABA_AR$ is increasing a constitutive, GABA-independent tonic current that is demonstrated in epsilon-containing recombinant $GABA_AR$s (Davies *et al.*, 1997; Thompson *et al.*, 2002). This constitutive tonic current could shunt excitatory currents in PBC neurons coming from the dendrites to the cell soma (see PBC neuron section above) and lead to decreased respiratory frequency or apneas. Also, expressing a constitutive, GABA-independent tonic current may attenuate the respiratory network's ability to respond to enhanced excitatory drive, such as during hypoxia. However, constitutive activity in native epsilon-containing $GABA_AR$s has not been demonstrated. Further, any tonic current that is induced by increasing epsilon subunit expression may be offset by the concomitant decrease in delta-containing $GABA_AR$s that also mediate a tonic current (see Chapter 2). While strong evidence shows that epsilon subunits play a role in respiratory control, further studies are needed to carefully

determine the extent of influence epsilon subunit expression (and changes in expression) have in relation to overall respiratory output and neuronal excitability.

IV. $GABA_AR$ during pregnancy

$GABA_AR$ subunit changes during pregnancy when central allopregnanolone levels are increased

Pregnancy is a normal, essential, physiological process during which neurons are exposed to high central concentrations of neurosteroids for extended time periods: brain neurosteroid concentrations increase up to 100-fold (Bayliss *et al.*, 1987) and allopregnanolone levels increase 3-fold (Concas *et al.*, 1998). While pregnancy is associated with changes in the sensitivity of $GABA_ARs$ in the maternal brain to various drugs (Majewska *et al.*, 1989), our data and others (Hengen *et al.*, 2012) suggest that functional $GABA_ARs$ in respiratory-related brainstem regions are expressed during pregnancy because the GAB_AA agonist, muscimol, effectively inhibits neurons. Thus, sensitivity to positive allosteric modulation must change to maintain adequate neuronal excitability. One likely mechanism is altering $GABA_AR$ subunit composition. In pregnant rats, alpha-5 (Follesa *et al.*, 1998), delta (Maguire and Mody, 2008), and gamma-2 (Follesa *et al.*, 2002; Maguire and Mody, 2008) subunit expression decreases. On the other hand, alpha-1 subunits increase (Concas *et al.*, 1999; Fenelon and Herbison, 1996; Follesa *et al.*, 1998). These data suggest that changing $GABA_AR$ subtype expression maintains neuronal function during pregnancy.

Epsilon subunits increase in respiratory-related neurons during pregnancy: Recently, our group showed that PBC-region neurons increase epsilon subunit expression during late pregnancy, presumably to protect breathing from increased allopregnanolone concentrations (Hengen *et al.*, 2012). During late

pregnancy, respiratory motor output on the phrenic nerve continues significantly longer following sequential pentobarbital injections in pregnant rats compared to non-pregnant female rats (Hengen *et al.*, 2012). Specifically, respiratory frequency is preserved rather than phrenic burst amplitude, suggesting PBC neurons increase epsilon subunit expression. Consistent with this hypothesis, neurons in the PBC-region of acutely isolated medullary slices from pregnant rats are more resistant to bath-applied pentobarbital compared to neurons from non-pregnant female and male rats (Hengen *et al.*, 2012). Also, epsilon subunit immunoreactivity increases in the PBC region in pregnant rats compared to non-pregnant female and male rats (Hengen *et al.*, 2012). It remains unknown whether: 1) increasing epsilon subunit expression during pregnancy is unique to neurons in the PBC region or if other respiratory-related neurons, such as XII motoneurons, increase epsilon subunit expression during pregnancy, 2) delta subunits decrease in respiratory-related neurons (similar to the hippocampus) or 3) epsilon subunit expression can be induced by pharmacologically mimicking pregnancy. Briefly, we found that XII motoneurons also increase resistance to pentobarbital and allopregnanolone, suggesting increased epsilon subunit incorporation into $GABA_AR$ during pregnancy. Additionally, our data show that delta subunit mRNA decreases in XII motoneurons (Chapter 2) and that epsilon subunit expression can be induced with daily injections of a progesterone analogue or allopregnanolone (Appendix).

V. Critical period in respiratory control development

Clinical and physiological significance of the critical period

Potential clinical significance: Preterm human infants (born at <34 gestational weeks) have strikingly immature respiratory control systems and often suffer from central apneas (*i.e.*, apnea of

prematurity) that cause blood oxygen desaturation and lead to bradycardias (Raju, 2012; Vergales *et al.*, 2013). Apnea of prematurity is outgrown by or before 40 weeks gestational age, thereby suggesting a period of days-to-weeks when the respiratory control system develops rapidly. A detailed analysis of neurodevelopmental events suggests that a G196 human fetus (early third trimester) corresponds most closely to P12-13 rat (Clancy *et al.*, 2007). Interestingly, the rat respiratory control system undergoes a period of rapid development between P11-P15 (for review: Wong-Riley and Liu, 2008; Wong-Riley *et al.*, 2013). The critical period in respiratory control development represents a shift towards expressing the adult phenotype in receptor subunit composition and neurotransmitter concentrations, but also a window of increased vulnerability to respiratory control. Therefore, understanding the endogenous mechanisms that create abrupt developmental changes and most importantly, stabilize breathing could have powerful clinical implications for pre-term infants.

Physiological significance: During the critical period in respiratory control development, normoxic ventilation and the hypoxic ventilatory response are significantly different compared to other days during the second and third postnatal weeks. In young rats, respiratory frequency gradually increases from P0 until peaking at P13; followed by a gradual decrease until P21 (Liu *et al.*, 2006). In P13 rats there is also an abrupt increase in minute ventilation to oxygen consumption and to CO_2 production ratios in normoxia (Liu *et al.*, 2009). Additionally, the hypoxic ventilatory response is significantly attenuated from P12-P14, being the weakest at P13, compared to the rest of the first three postnatal weeks (Liu *et al.*, 2006). During acute hypoxia, P13 rats have an inadequate metabolic rate with compromised ratios of minute ventilation to oxygen consumption and to CO_2 production (Liu *et al.*, 2009). In contrast, these ratios remain stable during normoxia and hypoxia during the remaining second and third postnatal weeks (Liu *et al.*, 2009). These changes suggest that the massive rearrangement of receptor and subunit expression and neurotransmitter concentrations have distinct consequences to respiratory stability, especially during hypoxia. The inability to appropriately respond to hypoxia for a

brief time period may also represent increased vulnerability to sudden infant death syndrome (SIDS; Liu *et al.*, 2006, 2009).

Increased neuronal inhibition during the critical period in respiratory control development

Dominance of inhibitory neurotransmission: During the critical period, the balance of neuronal excitation *versus* inhibition appears to be shifted towards increased inhibition. For example, glutamate concentrations and NMDA receptor subunits NR1 and NR2A expression decrease significantly while GABA concentrations, and $GABA_B$, and glycine receptor expression increase in PBC, XII, and NTS neurons (Liu and Wong-Riley, 2002, 2005, 2010). Additionally, chloride transporters in the plasma membrane transition to the adult phenotype by switching from mainly NKCC1 at birth to predominately KCC2 at P12, when expression levels intersect in PBC, XII, and NTS neurons (Liu and Wong-Riley, 2012). Electrophysiological data also suggest increased inhibition in XII motoneurons at P12-13 because the: 1) amplitude and frequency of spontaneous EPSCs is significantly decreased while spontaneous IPSCs increased; 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 $GABA_A$Rs, the subunit composition switches from predominately expressing alpha-3 to alpha-1 in PBC and NTS neurons (Liu and Wong-Riley, 2004, 2006), likely improving the efficiency of inhibition by decreasing channel decay time (Bosman *et al.*, 2002; Wong-Riley *et al.*, 2013). These findings suggest that inhibitory neurotransmission is dominant during the critical period. However, excessively inhibiting respiratory-related neurons could decrease respiratory drive and diminish airway patency. Compensatory mechanisms within the respiratory control system to offset increased neuronal inhibition are unknown.

Allopregnanolone increases during the critical period: Another potential contributing factor to increased neuronal inhibition is the 3-4 fold increase in brain allopregnanolone levels during the critical

period (compared to P8 and P15 levels; Grobin and Morrow, 2001; Fig. 2). Our working hypothesis is that the respiratory control system compensates for elevated brain allopregnanolone levels by increasing epsilon subunit incorporation in $GABA_AR$s to become resistant to positive allosteric modulation. Medullary epsilon subunit mRNA is expressed and increasing in rats from E14-P12 (Pape *et al.*, 2009), however, the contribution of functional epsilon subunits in respiratory-related neurons and day-to-day changes in expression during the critical period are unknown. We found that epsilon subunit incorporation in $GABA_AR$s dynamically changes during the critical period in a region-specific manner (Chapter 3), demonstrating that epsilon subunits may play an important role in protecting breathing during development.

VI. Increased epsilon subunit expression protects neurons from excessive inhibition

Three physiological models suggest epsilon subunits are inserted into $GABA_AR$s to protect breathing from excessive allopregnanolone-dependent inhibition: hibernation, pregnancy, and the critical period in respiratory control development. Increasing epsilon subunit expression is an attractive neuroprotective strategy because it provides versatile resistance to positive allosteric modulators (pentobarbital, allopregnanolone, and ethanol) that is reversible, fast-acting, inducible, and brain-region specific with few apparent drawbacks. Overall, increasing epsilon subunit expression appears to protect breathing from excessive neuronal inhibition. The $GABA_AR$ subunit hypothesis highlights an endogenous protective mechanism that the respiratory control system utilizes under normal, physiological conditions.

VII. Ischemic neuroprotection

The respiratory control system must also have mechanisms in place to protect itself from pathophysiological conditions such as ischemia. Perinatal ischemia is an important physiological model to study because the perinatal period is a time of clearly defined risk. Two potential strategies for providing neuroprotection during pathological conditions are described below.

Providing neuroprotection from perinatal ischemia

Incidence and causes: Perinatal ischemia is a general term associated with the loss of blood flow to the CNS during the perinatal period; defined as starting from the 20th week of gestation through to the 28th postnatal day in humans (Raju *et al.*, 2007). Specific subtypes of perinatal ischemia include hypoxic-ischemic encephalopathy (oxygen deficiency in the whole brain), perinatal asphyxia (lack of oxygen to the fetus during labor and delivery), and perinatal stroke (*e.g.*, focal disruption of cerebral blood flow due to arterial or venous thrombosis). These pathological conditions lead to lack of oxygen (and glucose in some cases) to the brain and spinal cord, which initiates a cascade of events leading to neuronal damage and cell death. Perinatal ischemia is caused by a wide variety of clinical conditions, such as thrombosis (Nelson, 2007), perinatal ischemic stroke (Nelson, 2007), acute fetal circulatory collapse (Rennie *et al.*, 2007), placental insufficiency (Badr and Purdy, 2006), forceps application (Volpe, 1994), dysfunctional labor (Volpe, 1994), inappropriate use of maternal drugs causing pharmacologically-induced fetal respiratory depression (Volpe, 1994), birth asphyxia (Whitelaw and Thoresen, 2002), and respiratory or cardiac failure (Badr and Purdy, 2006). Perinatal stroke and birth asphyxia occur at rates of 1 per 2300-5000 and 9.4 per 1,000 live births, respectively (Legido *et al.*, 2000; Laugesaar *et al.*, 2007; Palsdottir *et al.*, 2007), while hypoxic-ischemic encephalopathy occurs at a rate of

1.4 per 1,000 live births (Palsdottir *et al.*, 2007). Perinatal ischemia can lead to life-long conditions such as motor disabilities, seizure disorders, cerebral palsy, and respiratory difficulties (Volpe 2001; Badr and Purdy, 2006; Sotero de Menezes and Shaw, 2006; Nelson 2007). In one study of 46 neonates with hypoxic-ischemic encephalopathy, 44% of surviving children had significantly delayed motor abilities (van Schie *et al.*, 2007).

Ischemic signaling cascade: Reduced blood flow to the brain impairs delivery of oxygen and glucose, which then reduces ATP availability, and initiates a cascade of events (Fig. 3). Energy depletion results in dysfunctional ATP-dependent ion gradients and ion exchangers causing cellular depolarization and excessive excitatory neurotransmitter release--extracellular glutamate concentration increases 3–10 fold during ischemia (Sanders *et al.*, 2007). Excitotoxic injury is further compounded since energy-dependent glutamate reuptake is compromised. Activation of postsynaptic glutamate receptors produces a transmembrane flux of sodium and calcium cations, which contributes to depolarization and neuronal excitation (Sanders *et al.*, 2007). Water passively follows sodium and calcium ion influx and contributes to brain swelling. Along with glutamatergic excitotoxicity and calcium ion influx, there is free radical attack and prolonged seizure activity, which causes further neuronal damage (Hagberg *et al.*, 2001). High intracellular calcium levels activate numerous signaling cascades that cause further tissue damage. Following excitotoxic injury and loss of synaptic connectivity, apoptosis or programmed cell death is initiated (Sanders *et al.*, 2007). Inflammation and apoptosis increase over hours to days after the initial ischemic event, and neurotrophic factors are downregulated (Hagberg *et al.*, 2001; van Bel and Groenendaal, 2008).

Challenges in translating experimental findings to the clinic: While the ischemic cascade in the brain is well understood (Hoyte *et al.*, 2004), translating a neuroprotective animal model to clinical practice is highly problematic (Rother, 2008). Various factors contribute to this difficulty because in animal studies, neuroprotective drugs are typically given in healthy rats shortly after (or prior to)

administering the ischemic insult (usually blood vessel occlusion) that results in a reproducible ischemic lesion. In contrast, neuroprotective drugs in clinical trials are given at various times following strokes that produced highly variable brain lesions in humans who may have significant co-morbidity. Also, brain reperfusion is usually well controlled in animal studies, whereas reperfusion in humans is left to chance (except for studies testing thrombolytic drugs). Also, it's difficult to attain therapeutic levels of neuroprotective agents within the poorly perfused brain tissue. Animal studies often use infarct volume as an outcome measure whereas human clinical trials focus on functional outcomes (Hussain and Schuaib, 2008). Accordingly, expectations and goals for neuroprotection research need to be adjusted to reflect these realities, particularly determining *neuronal function* post-ischemia rather than only infarct size. Although ischemic events during the perinatal period are unpredictable, a woman in labor represents a clearly defined time when the mother and fetus are at risk for ischemic events that tend to occur during delivery and early postnatal life. Thus, it may be possible to prophylactically administer a drug combination to women to provide neuroprotection for the fetus before, during, and after parturition. Alternatively, a neuroprotective drug combination could be developed that would be available during an otherwise healthy delivery for use at the first sign of an ischemic event.

Ideal characteristics of a neuroprotective agent: The ideal neuroprotective agent against perinatal ischemia should be easy to administer, rapidly absorbed, and able to cross the placental and fetal blood-brain barriers with minimal or no adverse side effects (Johnson and Turner, 2010). The ideal agent should also activate endogenous neuroprotective mechanisms, disrupt the ischemic cascade at multiple points, and provide long-lasting (>24 h) protection no matter if given before, during, or after an unexpected perinatal ischemic event (Johnson and Turner, 2010). Increased central allopregnanolone levels and DOR activation have many of these characteristics as they appear to be highly conserved, inducible mechanisms, which provide versatile neuroprotection.

Strategy 1: enhance brain allopregnanolone concentrations during pathological conditions

Increasing brain allopregnanolone levels appears to be a versatile, effective, endogenous protective mechanism. As discussed above, allopregnanolone concentrations increase in the physiological processes of hibernation, pregnancy and development. Additionally, allopregnanolone levels also increase under pathological conditions such as umbilical cord occlusion (Nguyen *et al.*, 2004), intrauterine growth restriction (Westcott *et al.*, 2008), and hypoxia (Billiards *et al.*, 2006). Central allopregnanolone levels increase in <1.0 h in response to umbilical cord occlusion (Nguyen *et al.*, 2004). Thus, brain allopregnanolone levels naturally and rapidly increase during pathological conditions when brain oxygen levels are decreased.

Pharmacologically increasing brain allopregnanolone levels appears to be a compelling strategy for improving neuronal function. For example, adult male rats subjected to transient, severe forebrain ischemia and treated with allopregnanolone periodically from 20 min to 72 h post-injury had significantly better cognitive function (spatial learning/memory; reference/working memory) three months after ischemia compared to sham rats (Morali *et al.*, 2011). Further, allopregnanolone administration provides neuroprotection against perinatal ischemia. Maternal allopregnanolone injection (3 mg/kg; SQ) one hour before neonates were subjected to *in utero* asphyxia significantly improved expression of long-term potentiation in the hippocampus in P5 mice (Fleiss *et al.*, 2012). Also, maternal allopregnanolone injection attenuated the asphyxia-induced increase in calcium channel expression in neonatal CA1 pyramidal neurons (Fleiss *et al.*, 2012), suggesting a potential mechanism for allopregnanolone-dependent neuroprotection. The augmentation of $GABA_AR$ activity by allopregnanolone appears to play an important role in protecting neurons. For example, in mouse brain slices *in vitro* allopregnanolone application decreased dopamine efflux and neuronal loss caused by OGD; an effect that was blocked by a selective $GABA_AR$ antagonist (Knight *et al.*, 2012). While these

reports demonstrate allopregnanolone-dependent neuroprotection in the highly ischemia-susceptible cortex and hippocampus, it is unknown whether allopregnanolone administration can provide neuroprotection to respiratory motor circuits that are required for blood-gas homeostasis. Our preliminary data suggest that medullary slices treated with allopregnanolone 2.0 h before a 20 min OGD exposure increases NTS and XII neuronal firing rates 1.0 h later compared to OGD-only treated slices *in vitro* (Johnson Lab, unpublished observations). Therefore, allopregnanolone is an attractive candidate for providing neuroprotection to respiratory motor circuits from perinatal ischemia.

Increased brain allopregnanolone levels induce epsilon subunit expression to protect breathing

Interestingly, in conditions when brain allopregnanolone levels increase, the expression of epsilon subunits in respiratory-related neurons also increases (*e.g.*, during pregnancy). Our preliminary data further suggest increased central allopregnanolone concentrations during pathological conditions also increase epsilon subunit expression. Specifically, one lipopolysaccharide injection (1.0 mg/kg; SQ) increases PBC-region neuronal resistance to positive allosteric modulators *in vitro* from only 13% of neurons in untreated rats to 54% of neurons in LPS-treated rats (n=2 P30 rats; 34 cells) (Johnson Lab, unpublished observations). Further, epsilon subunit mRNA is increased in medullas from adult male rats only 3 h post-LPS injection (Watters Lab, unpublished observations) and also after chronic, intermittent hypoxia for 14 days (Watters Lab, unpublished observations). Taken together, these data suggest the increased epsilon subunit expression is a compensatory mechanism to pathological conditions that pose a risk to respiratory control. While increasing brain allopregnanolone concentrations appears to be an important endogenous protective mechanism, it also appears to be linked to increased epsilon subunit expression. Our working hypothesis is that epsilon subunit expression specifically increases in respiratory-related neurons to protect breathing from allopregnanolone-dependent excessive inhibition.

Therefore, increasing epsilon subunit expression is an important component of endogenous neuroprotection.

Strategy 2: application of principles from extremophile vertebrates to ischemia-susceptible mammals by activating DOR

DOR activation appears to be an endogenous protective mechanism utilized by extremophile vertebrates such as hibernators (thirteen-lined ground squirrels, woodchucks) and hypoxia-resistant red eared slider turtles. Compared to ischemia-susceptible mammals, hibernators exemplify natural tolerance to oxygen-, blood-, or energy-deprivation (Drew *et al.*, 2001; Borlongan *et al.*, 2004). During hibernation, blood flow to the brain is severely reduced but neurons remain viable (Drew *et al.*, 2001) and cardiorespiratory function is still regulated during torpor (Drew *et al.*, 2007). Hibernation-induced neuroprotection is not simply due to colder brain temperatures, but appears to be due to increased resistance to ischemic conditions (Drew *et al.*, 2007; Bullard *et al.*, 1960). Therefore, hibernators must possess endogenous protective mechanisms to maintain neuronal function. DOR activation appears to be a key component of the hibernation cycle and to hypoxia resistance. For example, injections of Deltorphin-Dvariant (DOR agonist) or hibernating woodchuck plasma into mice prior to undergoing focal ischemia provided neuroprotection (Govindaswami *et al.*, 2008). Likewise, hypoxia-resistant red-eared slider turtles can hold their breath for up to 48 h (Musacchia, 1959). This ability is hypothesized, in part, to be due to endogenous DOR activation because hypoxia-resistant red eared slider turtles have greater DOR expression in the CNS compared to rats (Xia and Haddad, 2001). Further, endogenous DOR activation protects against NMDA-dependent excitotoxicity in anoxic turtle cortical slices (Pamenter and Buck, 2008). Therefore, increasing DOR expression and/or activating DOR in ischemia-susceptible mammals (*i.e.*, mice, rats, humans) may be a powerful tool to protect neurons from acute ischemic injury.

One strategy to activate DOR in ischemia-susceptible mammals is to administer a pharmacological agent for the treatment of ischemic damage. The goal of providing neuroprotection by influencing multiple complex signaling pathways simultaneously over different time frames may best be achieved with the introduction of pleiotropic drugs (*i.e.*, single drugs that produce multiple effects; Menger and Vollmar, 2007). DOR agonist drugs are pleiotropic because they disrupt several steps in the acute phases of the ischemic cascade (see asterisks in Fig. 3) via different mechanisms (Table 1). Although some mechanistic features may be tissue specific and species-specific, Table 1 illustrates DOR agonists' capacity to attenuate multiple deleterious ischemic events. In addition to protecting against acute excitotoxicity during ischemia, DOR activation attenuates signaling pathways that continue for hours to days after the initial ischemic event. For example, Tan-67 (DOR agonist) administration 24 h prior to OGD solution application reduces cell death in organotypic hippocampal cell cultures (Zhao *et al.*, 2006). Similarly, Tan67 administration 24 h prior to right middle cerebral artery occlusion reduces infarct size and improves functional outcome (Zhao *et al.*, 2006). Thus, DOR activation can induce neuroprotection lasting for at least one day, suggesting that DOR activation may induce a type of protective neuroplasticity. However, much less is known about DOR activation's capability to protect motor networks, especially in perinatal animals.

DOR activation provides neuroprotection in spinal motor networks

Since cortical and hippocampal tissues are highly sensitive to ischemia, most information on DOR-dependent neuroprotection is derived from studies on these tissues. With respect to motor networks, a robust literature on spinal cord ischemia in adults exists due to the problem of spinal cord ischemia occurring during surgical aortic aneurysm repair. In addition to several studies on ischemic preconditioning in spinal cord, DOR activation is neuroprotective in adult spinal cord. Intrathecal SNC80 (DOR agonist; 40 mM) protects against spinal cord ischemia administered 9 min later in adult rat lumbar

spinal cord (Horiuchi *et al.*, 2004). Forty-eight hours afterwards, hind limb motor function is improved and significantly more neurons are uninjured compared to sham rats (Horiuchi *et al.*, 2004). Although DOR activation is neuroprotective in mature spinal cords, it is important to understand how ischemia alters motor network function in younger mammals since perinatal ischemia causes significant morbidity with respect to motor function.

Albeit to a lesser extent than extremophile vertebrates, DORs are expressed and functional in the neonatal rat spinal cord (Attali *et al.*, 1990). Binding affinities for DORs in the rat brain or spinal cord are constant or increase from the first postnatal day (McDowell and Kitchen, 1986; Attali *et al.*, 1990; Szucs and Coscia, 1990) and DOR expression is postulated to increase 40 fold between neonates and adults (Milligan *et al.*, 1987). The location of DORs in the neonatal rat spinal cord is not known, but DOR immunoreactivity is located in the ventral horn of adult rat spinal cords (Mailly *et al.*, 1999). Thus, the substrate for DOR-dependent neuroprotection is present in the neonatal spinal cord, and may be located both pre- and postsynaptically in the ventral horn throughout development. The potential role of DOR activation in providing neuroprotection in the neonatal spinal cord during OGD exposure is largely not known.

To address this question in our laboratory, the neuroprotective effects of DOR activation on spinal respiratory motor circuits were studied in neonatal rat brainstem-spinal cord preparations. Instead of electrically evoking spinal motoneurons responses, we examined the effects of spinal OGD solutions on spontaneously-produced, quantifiable respiratory motor output on cervical and thoracic spinal ventral roots. We tested whether cervical and thoracic respiratory motor output are equally sensitive to OGD, and whether neuroprotection is provided in the following conditions: 1) sustained spinal DOR activation prior to and during spinal OGD, 2) brief spinal DOR activation several minutes prior to spinal OGD (*i.e.*, a form of neuroplasticity), and 3) spinal DOR activation following the onset of OGD exposure. For further discussion see Chapter 4.

VIII. REFERENCES

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Table 1: Potential mechanisms underlying DOR-dependent neuroprotection.

Ischemic Cascade Event	DOR-Dependent Action	Key Features	References
Mitochondrial Depolarization	K_{ATP} channel activation	Protects cultured cortical neurons against sodium azide-induced mitochondrial respiratory chain injury and maintains DOR levels.	Zhu et al., 2009
Na^+ Influx	Decreases Na^+ influx	Blocks voltage-gated Na^+ channels and reduces Na^+ influx via NMDA channels in cortical slices exposed to anoxia.	Chao et al, 2008, 2009
K^+ Efflux	Decreases K^+ efflux	Attenuates K^+ efflux from cortical slices exposed to anoxia or OGD via PKC-dependent, PKA-independent pathway. Inhibition of Ca^{2+} influx reduces activation of Ca^{2+}-activated K^+ (BK) channels in cortical slices exposed to anoxia.	Chao et al., 2007a,b Chao et al., 2007a
Ca^{2+} Influx	Decreases Ca^{2+} influx	Indirect evidence of reduced Ca^{2+} influx cortical slices exposed to anoxia. Hypoxia-induced Ca^{2+} influx reduced in adrenal medulla cells by decreasing voltage-gated Ca^{2+} currents.	Chao et al., 2007a Keating et al., 2004
Increased Glutamate Release	Inhibits glutamate release presynaptically	Decreased amplitude of AMPA EPSCs/EPSPs in lamina II of lumbar spinal cord slices without altering responses to pressure-ejected AMPA. Decreased frequency, but not amplitude, of mEPSCs in amygdala slices.	Glaum et al., 1994 Bie et al., 2009
Increased AMPA and NMDA Receptor Activation	Decreases NMDA-dependent currents	Reduces Na^+ influx via NMDA channels in cortical slices exposed to anoxia. Reduces NMDA currents during anoxia in turtle cortical slices.	Chao et al., 2009 Pamenter and Buck, 2008
Increased Free Radical Production	Decreases free radical release or impact	Hypoxic preconditioning attenuates decrease in antioxidant scavengers and increase in oxidant proteins via DOR-dependent mechanism in retinal cells. DOR agonist drug acts as free radical scavenger. DOR agonist and plasma from hibernating woodchuck reduces nitric oxide release in microglia cell culture via DOR mechanism.	Peng et al., 2009 Tsao et al., 1998 Govindaswammi et al., 2008

Figure 1: Pictorial description of key respiratory-related CNS regions. Respiratory rhythm is generated in the brainstem and transmitted to spinal motoneurons that innervate the diaphragm and intercostals muscles.

Fig. 1

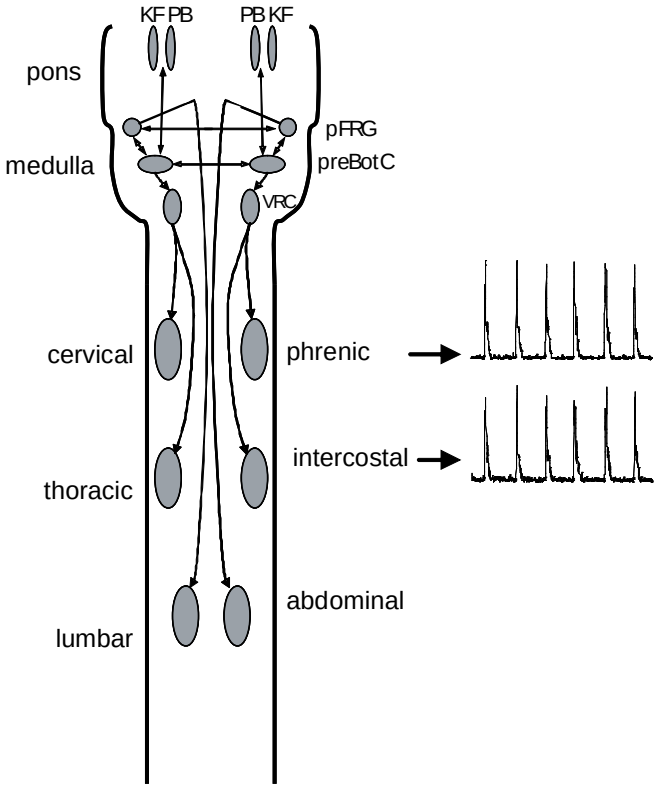

KF PB
PB KF
pons
pFRG
medulla
preBotC
VRC
cervical
phrenic
intercostal
thoracic
abdominal
lumbar

Figure 2: Allopregnanolone levels increase in the cerebral cortex at P10 and P14. On postnatal days 10 and 14, allopregnanolone levels abruptly increase. This time frame corresponds to the critical period in respiratory control development. Data are presented as ng/g (mean±S.E.M.) for 6–12 animals from five separate experiments. Overall ANOVA $P<0.001$; *$P<0.05$ for comparison of PD10 to PD7 and PD8. PD14 values were significantly different than PD15, PD16 and PD21 using Tukey's post hoc test. (from Grobin and Morrow, 2001; permissions requested.)

Fig. 2

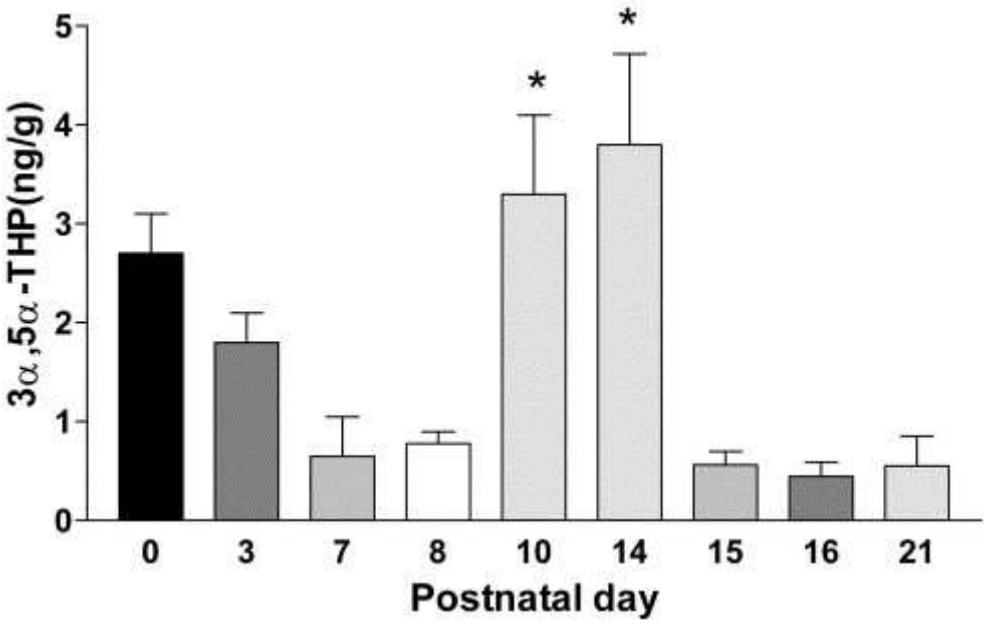
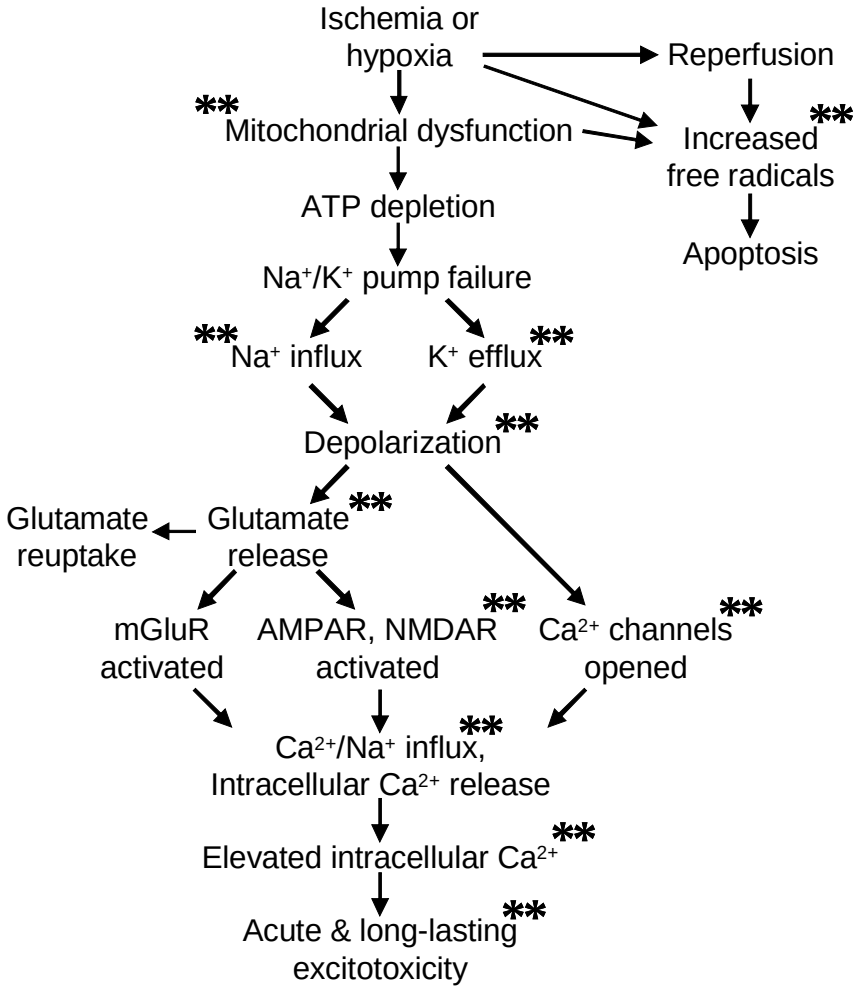


Figure 3: DOR activation disrupts the ischemic signaling cascade. Ischemia or severe hypoxia reduces ATP production and initiates a cascade of events that lead to neuronal damage or death. Experimental evidence shows that DOR activation has to the capacity to attenuate or block several steps in the cascade. ** Indicates a step that is disrupted by DOR activation (from Johnson and Turner, 2010; permissions requested)

Fig.3


Ischemia or hypoxia
Reperfusion
Mitochondrial dysfunction
Increased free radicals
ATP depletion
Apoptosis
Na+/K+ pump failure
Na+ influx
K+ efflux
Depolarization
Glutamate reuptake
Glutamate release
mGluR activated
AMPAR, NMDAR activated
Ca2+ channels opened
Ca2+/Na+ influx, Intracellular Ca2+ release
Elevated intracellular Ca2+
Acute & long-lasting excitotoxicity

Chapter 2:

Increased resistance of $GABA_A$ receptors to pentobarbital and allopregnanolone in hypoglossal motoneurons during late pregnancy suggests increased $GABA_A$ epsilon subunit expression

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I. ABSTRACT

Allopregnanolone is increased centrally during late pregnancy, thereby creating a risk for excessive neuronal inhibition because allopregnanolone is a positive allosteric modulator of $GABA_A$ receptors ($GABA_A$Rs). We hypothesized that XII motoneurons compensate by increasing inclusion of epsilon subunits in $GABA_A$Rs because the epsilon subunits confer resistance to positive allosteric modulators, such as allopregnanolone and pentobarbital. Thus, thin medullary slices were isolated from G18 pregnant rats, adult male and virgin female rats. Silicon multichannel electrodes extracellularly recorded spontaneous action potentials within the XII motor nucleus. To pharmacologically test for epsilon subunits, slices were exposed to pentobarbital (200-300 μM) or allopregnanolone (1.0 μM). XII motoneuron firing rates from male and female rats decreased to 51-62% of baseline during pentobarbital (300 μM) application and to 28% of baseline in male rats during allopregnanolone application. In contrast, XII motoneuron firing rates from G18 pregnant rats increased to 120 ± 29% and 146 ± 17% of baseline during pentobarbital and allopregnanolone applications, respectively. To test whether this resistance was due to changes in XII motoneuron $GABA_A$R subunit composition, slices were bathed in a high Mg^{2+}/low Ca^{2+} solution to block synaptic transmission. In synaptic blockade solution, male and G18 pregnant rat XII motoneuron firing rates decreased to 52 ± 17% and 59 ± 9% of baseline, respectively, during pentobarbital (300 μM) application. The pentobarbital-dependent decrease in G18 pregnant rat XII motoneurons was likely non-specific since it was not blocked by bicuculline, or bicuculline/strychnine co-application. During allopregnanolone (1.0 μM) application in synaptic blockade solution, male rat XII motoneuron firing rates decreased to 72 ± 6% of baseline while G18 pregnant rat firing rates were 149 ± 17% of baseline. Epsilon subunit mRNA expression was not different between male and pregnant rats, although respiratory-related brainstem regions did have significantly increased epsilon subunit mRNA compared to non-respiratory related areas. These data support the hypothesis

that $GABA_AR$s increase epsilon subunit incorporation to prevent excessive allopregnanolone-dependent inhibition during pregnancy.

II. INTRODUCTION

The respiratory control system must dynamically adjust excitatory and inhibitory neuronal signaling to maintain blood-gas homeostasis during physiological challenges. During pregnancy, the respiratory control system often needs to compensate for progressive weight gain, upward diaphragm displacement, and estrogen-induced mucosal edema (Venkata and Venkatashiah, 2009). Furthermore, during pregnancy, central progesterone concentrations increase up to 100-fold (Backstrom *et al.*, 2003) and contribute to increasing central allopregnanolone concentrations by 3-fold (Concas *et al.*, 1998). Allopregnanolone enhances inhibitory signaling by acting as a positive allosteric modulator (increases chloride ion influx) on $GABA_AR$s (Kokate *et al.*, 1994; Reddy *et al.*, 2004). Since $GABA_AR$s are widely expressed in respiratory neurons (Koshiya and Guyenet, 1996; Paton and Richter, 1995), elevated central allopregnanolone levels could cause excessive neuronal inhibition and disrupt breathing (Ren and Greer, 2006 a,b). Since pregnant animals breathe without difficulty (Bayliss and Millhorn, 1992), the mechanisms underlying $GABA_AR$ resistance to positive allosteric modulators within the respiratory control system are not known.

Our group recently hypothesized that the respiratory control system actively adjusts during pregnancy to avoid excessive inhibition by altering $GABA_AR$ subunit composition to confer insensitivity to positive allosteric modulation (Hengen *et al.*, 2009, 2011, 2012). $GABA_AR$ subunit composition determines the receptor's subcellular localization and functional characteristics. $GABA_AR$s are

pentameric (Bonnert *et al.*, 1999) and typically composed of two alpha subunits, two beta subunits, and one gamma subunit. Inclusion of other subunits (delta, epsilon, theta, pi or rho) modulates GABA affinity, GABA efficacy, channel gating properties, and sensitivity to positive allosteric modulators (Sieghart and Sperk, 2002). Inclusion of the epsilon subunit confers resistance to positive allosteric modulation by pentobarbital (Irnaten *et al.*, 2002) and allopregnanolone (Davies *et al.*, 1997). In contrast, $GABA_A$Rs containing delta subunits have increased sensitivity to positive allosteric modulators (Olsen *et al.*, 2007; Hevers and Lüddens, 1998). During pregnancy, epsilon subunit expression is increased in ventral respiratory column neurons (Hengen *et al.*, 2012) while delta subunit expression is decreased in hippocampal neurons (Maguire and Mody, 2008). However, little is known about $GABA_A$R subunit expression in adult respiratory motoneurons during pregnancy. Hypoglossal (XII) motoneurons are ideal to study because their function with respect to respiratory motor control is well known. XII motoneurons integrate inputs from the medulla, pons and cortex (Peever *et al.*, 2002) and activate tongue muscles during inspiration to maintain upper airway patency (Remmers *et al.*, 1978; Fregosi and Fuller, 1997; Wrobel *et al.*, 2010). Consequently, failure to maintain appropriate excitation and inhibition within XII motoneurons may cause apnea, ataxic breathing, and blood-gas disturbances. We hypothesized that XII motoneurons increase epsilon subunit incorporation in $GABA_A$R during pregnancy to confer resistance to positive allosteric modulators, such as allopregnanolone.

To address these questions, functional epsilon-expressing $GABA_A$Rs were demonstrated *in vitro* with extracellular multichannel recordings of spontaneous XII motoneuron activity in medullary slices from pregnant rats, adult male and female rats during pentobarbital or allopregnanolone applications. To test whether resistance to pentobarbital or allopregnanolone is a property of XII motoneurons *versus* a property of the local network, experiments were repeated using high Mg^{2+}/low Ca^{2+} solution to block synaptic transmission (Johnson *et al.*, 1994). Finally, we measured epsilon and delta mRNA using

semiquantitative polymerase chain reaction (qRT-PCR) in tissue punches from XII motor nucleus and other medullary regions from male and pregnant rats. Taken together, our results suggest XII respiratory motoneurons from pregnant rats express functional epsilon subunit-containing $GABA_A$Rs that are more resistant to positive allosteric modulators.

III. METHODS

In vitro electrophysiological recordings

Experimental procedure: 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 139 Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were used, including 63 adult males (3-4 mo), 9 adult virgin females in diestrus (3-4 mo), 9 post-partum (30 d post-partum) females, and 58 pregnant rats (Table 1). To determine whether virgin female rats were in diestrus, rats were briefly anesthetized with 2-3% isoflurane (balance oxygen) for 1-3 min to allow sampling of vaginal cells with a saline-soaked cotton swab. If the vaginal smear contained primarily leukocytes, rats were considered to be in diestrus (Marcondes *et al.*, 2002) and used that day for experiments. Pregnant rats were studied on gestational days 16-18 (G16-G18) because previous studies show changes in $GABA_A$R epsilon and delta subunit expression at this gestational time point (Hengen *et al.*, 2012; Maguire and Mody, 2008). In the rest of the manuscript, pregnant rats will be referred to as G18 for simplicity because > 80% of pregnant rats used were exactly G18 on the day of the experiment.

Rats were deeply anesthetized with 5% isoflurane (O_2 balance) until the toe-pinch response was abolished. Medullas were dissected by making transverse cuts caudally at C1 and rostrally at the pontomedullary junction. A series of 3-4 coronal medullary slices (400 µm thick) containing the XII motor nucleus were cut in ice-cold 3 mM KCl artificial cerebrospinal fluid solution (aCSF) with a vibrating microtome (Campden Instruments, Layfayette, IN, USA). Upon removal, slices were immediately placed into an interface recording chamber (Warner Instruments, Hamden, CT, USA) and subfused with warm aCSF (8 ml/min). Slices were maintained at 37°C by a temperature controller (Harvard Apparatus, Holliston, MA, USA). Spontaneous neuronal activity was recorded from the XII motor nucleus in 3-4 medullary slices from the same rat using four 16-channel extracellular silicon electrode arrays (model a4x4-3mm100-177, Neuronexus, Ann Arbor, MI, USA). Electrodes were composed of four shanks, each with four recording sites. 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. Electrodes were inserted into medullary slices at a ~45° angle such that the array spanned the entire XII nucleus from lateral edge to midline in the slice (Fig. 1). Slices were allowed to equilibrate in aCSF at 37°C with electrodes inserted for 60-90 min before recording baseline activity for 30 min. The composition of the aCSF was (in mM): 120 NaCl, 26 $NaHCO_3$, 20 glucose, 2.0 $MgSO_4$, 1.0 $CaCl_2$, 1.25 Na_2HPO_4, 7.0 KCl (potassium levels were increased to increase the number of active neurons). In separate experiments, high Mg^{2+}/low Ca^{2+} aCSF solution composed of (in mM): 120 NaCl, 21 $NaHCO_3$, 25 glucose, 1.0 $MgSO_4$, 4.0 $MgCl_2$, 1.0 $MgSO_4$, 4.0 $MgCl_2$, 0.2 $CaCl_2$, 0.5 Na_2HPO_4, 9.0 KCl was used to achieve synaptic transmission blockade (Johnson *et al.*, 1994).

Experimental Protocol: Following equilibration (60-90 min) and baseline recordings (30 min), pentobarbital or allopregnanolone (dissolved in dimethylsulfoxide [DMSO]) were added to the aCSF solution that subfused the slices for 45 min to determine neuronal sensitivity to positive allosteric

modulation. To test whether drug-induced changes in neuronal firing rates occurred through $GABA_AR$ or glycine receptors, bicuculline ($GABA_AR$ antagonist) or strychnine (glycine receptor antagonist) were applied before and during pentobarbital or allopregnanolone exposure. During the last 15 min of some experiments, muscimol ($GABA_AR$ agonist) was applied to the slices to confirm the presence of functional $GABA_ARs$ in pentobarbital- or allopregnanolone-resistant neurons. To examine possible artifacts due to drift across the duration of recordings, time control experiments were run for 240 min with no drug application.

Experimental Drugs: Pharmacological compounds used in this study included: sodium pentobarbital (200-300 μM; Fort Dodge Animal Health, Fort Dodge Iowa, USA); allopregnanolone (1.0 μM; Tocris Bioscience, Ellisville, MO, USA); muscimol (20 μM; Tocris Bioscience); bicuculline (100 μM; Tocris Bioscience); and strychnine (5 μM; Sigma Chemical Company, St. Louis, MO, USA).

Electrophysiological data analysis

Raw data were processed as described previously (Hengen *et al.*, 2009). Individual neurons were identified using Principal Component Analysis (Adamos *et al.*, 2008). Waveforms recorded on multiple, adjacent channels were counted only once. For channels containing multiple waveforms, neuronal assignments were made based on eigenvector cluster overlap, waveform shape, and cross-correlation analysis. Cross-correlation analysis was used to determine whether waveforms were neuronal and to test whether waveforms were from one or more neuron. Neuronal activity was averaged in 5-min bins and normalized to the mean firing rate during the 30-min baseline period 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 Hz, absence of action potentials for >10 consecutive min during the 30-

min baseline period, or a consistently decreasing firing rate was observed during the 30-min baseline period to <50% of the normalized value. Individual bins were discarded if the absolute firing rate was >500 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 min). Finally, if one cell was an outlier based on Grubb's outlier test for three consecutive 5-min bins during the last 15 min of drug application then all data for that cell were discarded (n=5 cells total). Based on these criteria, 11.8% of waveforms and 0.15% of data bins were discarded.

Electrophysiological data statistical analysis

To analyze changes in neuronal firing rates over time, normalized neuronal firing rates (averaged into 5-min bins) were log-transformed and individual rats were stratified into four categories: time controls (male, female and pregnant rats pooled), and three groups with drug application: virgin female, pregnant female and male rats. Data were analyzed with a mixed effect linear model to evaluate the effects of time and stratum on neuronal activity where fixed effects were condition, time, and their interaction. Random effects were rat type and neurons nested within rats. Group differences were tested with an F test. Specifically, the model was of the form: $Y_{ij} + X_{ij}\beta + u_{ij} + v_i$, where Y_{ij} represents the log neuronal activity measured from cell j within rat i, X_{ij} is a $t \times p$ design matrix of p-1 covariates for all t time measurements, which includes a term for the intercept, indicators for stratum classification (with time controls as the reference level), time at which the measurement was taken, and interactions between time and stratum. For every cell monitored for each rat, there were t = 31 (Figs. 2A, 2B) or 22 (Figs. 3-7, panels A, B) 5-min bins of normalized average neuronal firing rates. Hypothesis tests corresponding to differences in neuronal activity among strata were adjusted for multiple comparisons

using Bonferroni-corrected significance levels when necessary. Such contrasts were evaluated through the use of approximate Wald statistics. All analyses were conducted in R (R Development Core Team 2010), and the linear mixed effects model was fit using the *lmer* package (Bates and Maechler, 2010). The total number of neurons per condition was used as the number of independent samples for relevant statistical tests and calculation of S.E.M. All data are reported as means ± S.E.M.

To analyze the distribution of XII motoneuron resistance to pentobarbital or allopregnanolone, 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). $P<0.05$ was considered statistically significant for these data. Data are shown in cumulative histograms (Figs. 2C, 3C, 5C, 6C).

Epsilon and delta subunit mRNA quantification: RT-qPCR

Experimental protocol: A total of 12 male rats and 7 pregnant rats were used for mRNA quantification (see Table 1 for the number of samples included in the data set for each region). Medullas were isolated by making transverse cuts caudally at C1 and rostrally at the pontomedullary junction. A series of 5-6 coronal medullary slices containing the XII motor nucleus (400 µm thick) were cut in ice-cold 3 mM KCl aCSF with a vibrating microtome (Campden Instruments, Layfayette, IN, USA). Upon removal, slices were immediately placed into a dissecting dish and bathed in ice-cold 3 mM KCl aCSF. The following brain regions were dissected from the slice under a microscope (10x) to form 7 samples from each rat: XII nucleus, nucleus tractus solitarius (NTS), ventral respiratory column (VRC), the PBC-region (just caudal to the nucleus ambiguus) the midline region, and the dorsolateral edge of the slices (see Fig. 8A). A cortical sample was also taken from each rat. Tissue was homogenized in Tri-Reagent (Sigma, St. Louis, MO, USA), and total RNA was harvested according to the manufacturer's protocol.

Reverse transcription PCR (RT-PCR) was performed using 1.0 mg of total RNA as a template for the reverse transcription reaction using random hexamers and ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitative RT-PCR was conducted by monitoring in real-time the increase in fluorescence of the SYBR-GREEN dye using the TaqMan 7300 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Primer specificity was assessed through NCBI BLAST analysis prior to use, and all dissociation curves had a single peak with an observed Tm consistent with the intended amplicon sequences. Primer efficiency was calculated through the use of serial dilutions and construction of a standard curve.

Primer sequences for the following *rattus norvegicus* genes were used:

18s F: 5' AAC GAG ACT CTC GGC ATG CTA A 3'
18s R: 5' CCG GAC ATC TAA GGG CAT CA 3'
Epsilon F: 5' TGG AGC CTC AGC CTA GTG GAA AGA 3'
Epsilon R: 5' GGC GCA GTT TAT GGT CGT AGT TGC 3'
Delta F: 5' GCA TCC GCA TCA CCT CCA CA 3'
Delta R: 5' AGG AGG ACA ATG GCG TTC CT 3'

Epsilon and delta subunit mRNA data analysis

Epsilon and delta subunit gene expression data were analyzed based on a relative standard curve method, as specified by Applied Biosystems. All samples were run in duplicate, averaged, and interpolated onto previously run standard curves for each primer set to account for differences in primer efficiency. Values were then normalized to 18S. If the normalized gene expression data for an individual sample is greater than two standard deviations from the mean, the sample was excluded as an outlier.

Epsilon and delta subunit mRNA statistical analysis

Statistical analysis for epsilon and delta subunit mRNA were run on the normalized values data. Statistical analysis on epsilon and delta subunit mRNA expression failed equal variance and/or normality tests; therefore, data were transformed logarithmically before statistical analysis, but data were still reported as fold changes (Figs. 8, 9). Statistical significance was determined using a Two-Way ANOVA with Tukey post-hoc test (Sigma Stat, San Jose, CA, USA). Differences were considered significant if $p<0.05$. All values are expressed as means ± S.E.M.

IV. RESULTS

Time control experiments: XII motoneurons have stable spontaneous firing rates in different solutions

To confirm that XII motoneuron spontaneous activity remained stable for the duration of our experiments, slices from adult male (n=3 rats; 39 cells), adult virgin female (n=4 rats, 62 cells), G18 pregnant female (n=4 rats, 46 cells) and post-partum females (n=4 rats, 18 cells) were subfused for 4 h in standard 7 mM KCl aCSF. All groups produced stable activity with average XII motoneuron firing rates at 109 ± 7% of baseline values at the end of the 3-h time control experiments ($p>0.05$; Fig. 2A). Thus, the data were pooled together to form the time control data for later comparisons.

In separate, similar experiments to test whether XII motoneuron spontaneous activity was stable under conditions of synaptic blockade, slices from adult male (n = 8 rats; 98 cells) and pregnant rats (n=7 rats, 54 cells) were subfused with high Mg^{2+}/low Ca^{2+} aCSF solution for 3 h. There were no differences between male and pregnant rat time controls ($p>0.05$), so data were pooled. XII

motoneuron average firing rates were 142 ± 13% of baseline values at the end of the 3-h recording period (Fig. 3A).

In drug application studies, the vehicle DMSO was used to dissolve allopregnanolone and bicuculline. Therefore, control experiments were performed to test whether DMSO (0.01% solution) application changed XII motoneuron firing rates compared to aCSF time controls. XII motoneuron firing rates were unaltered for 3 h in standard aCSF with 0.1% DMSO (n=5 rats, 53 cells), and was not different compared to standard aCSF time controls ($p>0.05$). At the end of vehicle time control experiments, average XII motoneuron firing rates were 123 ± 16% of baseline values (Fig. 5A). The same was true for XII motoneuron firing rates in high Mg^{2+}/low Ca^{2+} synaptic blockade solution (n=8 rats, 57 cells; $p>0.05$). At the end of vehicle time control experiments, under synaptic blockade conditions, average XII motoneuron firing rates were 155 ± 33% of baseline values ($p>0.05$ compared to high Mg^{2+}/low Ca^{2+} time control experiments; Figs. 4A, 6A, 7A). Thus, in experimental groups containing allopregnanolone or bicuculline experiments, DMSO vehicle control data were pooled with the corresponding time control data.

Muscimol (20 μM) was applied during the final 30 min of a subset of time control experiments to confirm that $GABA_AR$ activation decreased spontaneous XII motoneuron activity. Across all time control groups, muscimol application decreased firing rates by ≥ 50% in 96.7% of XII motoneurons (88 of 91 neurons).

Pentobarbital resistance is increased in XII motoneurons from pregnant rats

To test pentobarbital sensitivity in XII motoneurons in medullary slices from adult male (n=6 rats; 105 cells), adult virgin female (n=5 rats, 64 cells), and pregnant female (n=7 rats, 78 cells) rats, 200

μM and 300 μM pentobarbital were applied sequentially for 45 min each. To confirm the presence of functional $GABA_A$Rs, muscimol (20 μM) was applied for the last 30 min of the experiment. XII motoneuron firing rates recorded from adult male and virgin female rats during 200 μM pentobarbital application steadily decreased to 51 ± 7% and 75 ± 9% of baseline, respectively, during the last 15 min (Figs. 2A, 2B; $p<0.003$ compared to time controls and G18 pregnant rats). In contrast, XII motoneurons from G18 pregnant rats were more resistant to pentobarbital and average firing rates increased to 111 ± 13% of baseline (Figs. 2A, 2B; $p = 0.05$ compared to time controls). Similarly, firing rates from male and female rats decreased to 31 ± 4% and 44 ± 6% of baseline, respectively, during the 300 μM pentobarbital application (Figs. 2A, 2B; $p<0.002$ compared to time controls and G18 pregnant rats). On the other hand, XII motoneurons from G18 pregnant rats were more resistant to 300 μM pentobarbital, with average firing rates during the last 15 min at 135 ± 30% of baseline (Figs. 2A, 2B; $p<0.002$ compared to time controls). To test whether the increase in pentobarbital resistance in pregnant rats is reversible following delivery of pups, the same experiments were repeated with 30 d post-partum female rats (n=5 rats, 70 cells). XII motoneuron firing rates decreased steadily during 200 μM and 300 μM pentobarbital application to 85 ± 16% and 48 ± 7% of baseline, respectively (data not shown). Thus, the increase in pentobarbital resistance during pregnancy appears to be reversible. In pentobarbital-resistant XII motoneurons from G18 pregnant rats, muscimol (20 μM) application decreased the average firing rate from 297 ± 13% to 31 ± 1% of baseline. Additionally, 93% of pentobarbital-resistant neurons tested for muscimol sensitivity were inhibited by ≥ 50% (Fig. 2A).

To determine the percentage of XII motoneurons that increased resistance to pentobarbital during pregnancy, the distribution of each XII motoneuron average firing rate during the last 15 min of 300 μM pentobarbital application was analyzed by constructing cumulative histograms. Compared to the curves for data from male and female rats, the curve from G18 pregnant rats was significantly right-

shifted ($p<0.001$) indicating a greater percentage of XII motoneurons from G18 pregnant rats were resistant to pentobarbital (Fig. 2C).

Since pentobarbital-resistant cells were a subpopulation of XII motoneurons, average firing rates from the last 15 min of 300 μM pentobarbital application were categorized into sensitive (<40% of baseline), intermediate (40-80% of baseline) or resistant (>80% of baseline) groups. During the last 15 min of 300 μM pentobarbital application, 70% of neurons from male, and 57% of neurons from female rats were sensitive to pentobarbital, while in G18 pregnant female rats 36% of neurons were pentobarbital-sensitive (Fig. 2D). With respect to pentobarbital-resistant XII motoneurons, only 8-16% of neurons from male and female rats, respectively, were resistant. In contrast, 40% of neurons from G18 pregnant rats were resistant; representing a 2-4 fold increase in pentobarbital resistant cells during pregnancy (Fig. 2D). In subsequent experiments, only male and G18 pregnant rats were studied, because pregnant rats are significantly more pentobarbital-resistant than male or virgin female rats.

Pentobarbital sensitivity under synaptic blockade conditions

To test whether pentobarbital sensitivity occurs on the XII motoneuron itself rather than through local network effects, slices from adult male (n = 8 rats; 72 cells) and G18 pregnant rats (n=8 rats, 72 cells) were subfused with synaptic blockade aCSF solution, followed by 300 μM pentobarbital application for 45 min in synaptic blockade aCSF solution (300 μM was the most effective dose in the previous experiments; Figs. 3A, 3B). XII motoneuron firing rates from adult male rats decreased to 52 ± 6% of baseline (Figs. 3A, 3B). Surprisingly, XII motoneurons from G18 pregnant rats also decreased firing rates to 59 ± 9% of baseline (Figs. 3A, 3B; $p>0.05$ compared to male rats; $p<0.001$ compared to time controls). Analysis of average firing rate distribution from each XII motoneuron did not indicate a shift

pentobarbital resistance during pregnancy in cumulative histogram curves (Fig. 3C, $p>0.05$). Categorizing XII motoneurons from male and G18 pregnant rats into sensitive, intermediate, and resistant groups showed that 49% and 61% of XII motoneurons from male and G18 pregnant rats, respectively, were sensitive to pentobarbital. Further, only 25% of XII motoneurons from both male and pregnant rats were resistant to pentobarbital. Muscimol sensitivity was tested in 20 pentobarbital-resistant neurons, and 5 neurons were also resistant to muscimol.

To further characterize these results, we tested whether pentobarbital sensitivity in XII motoneurons from G18 pregnant rats in synaptic blockade aCSF solution was due to positive allosteric modulation of $GABA_A$Rs. Thus, slices from G18 pregnant rats (n=3 rats, 23 cells) were obtained and subfused with synaptic blockade aCSF solution. After the baseline period, bicuculline (100 μM) was applied for 20 min before co-application of pentobarbital (300 μM) for 45 min. XII motoneuron firing rates decreased to 59 ± 9% of baseline during the last 15 min of pentobarbital application (Figs. 4A, 4B $p<0.0005$ compared to time controls). To test whether pentobarbital sensitivity was mediated through either major inhibitory chloride channel, bicuculline (100 μM) and strychnine (5 μM) were applied 20 min before and during pentobarbital (300 μM) co-application (n=3 rats, 26 cells). Again, XII motoneuron firing rates decreased to 58 ± 7% of baseline during the last 15 min of pentobarbital application (Figs. 4A, 4B $p<0.006$ compared to time controls). Thus, under conditions of synaptic blockade, pentobarbital sensitivity in XII motoneurons from G18 pregnant rats did not appear to be due to positive allosteric modulation of $GABA_A$Rs or glycine receptors and was likely due to non-specific pentobarbital effects.

Allopregnanolone resistance is increased in XII motoneurons from G18 pregnant rats

To test whether XII motoneurons from pregnant rats were resistant to allopregnanolone, slices from adult male (n=8 rats; 89 cells) and G18 pregnant rats (n=6 rats, 85 cells) were subfused with standard aCSF solution during baseline followed by a 1.0 μM allopregnanolone application for 45 min. XII motoneuron firing rates recorded from adult male rats decreased to 72 ± 6% of baseline during the last 15 min of allopregnanolone exposure (Fig. 5A, B; $p<0.0001$ compared to time controls and pregnant rats). In contrast, XII motoneurons from pregnant rats increased firing rates to 149 ± 17% of baseline (Figs. 5A, 5B; $p=0.021$ compared to time controls, [not significant]). Compared to male rats, the distribution of firing rates from G18 pregnant rats was significantly right-shifted ($p<0.001$) indicating a greater percentage of XII motoneurons from G18 pregnant rats were resistant to allopregnanolone (Fig. 5C). In male rats, 32% of neurons were allopregnanolone-sensitive, while in G18 pregnant rats allopregnanolone sensitivity was found in only 12% of neurons (Fig. 5D). Further, 40% of neurons from male rats are allopregnanolone-resistant, while resistance increased to 68% of neurons from G18 pregnant rats (Fig. 2D).

Allopregnanolone resistance occurs in XII motoneurons rather than the network

To test whether allopregnanolone resistance in pregnant rat XII motoneurons was due to changes in XII motoneuron $GABA_AR$ subunits, slices from adult male (n=8 rats; 76 cells) and G18 pregnant rats (n=7 rats, 53 cells) were subfused with synaptic blockade aCSF solution, followed by allopregnanolone application (1.0 μM; 45 min), and then by muscimol (20 μM) for 15-30 min to demonstrate functional $GABA_ARs$ in resistant neurons. XII motoneuron firing rates in adult male rats decreased to 54 ± 7% of baseline during allopregnanolone application (Figs. 6A, 6B; $p<0.001$ compared

to time controls and G18 pregnant rats). In contrast, XII motoneurons from G18 pregnant rats increased firing rates to 146 ± 17% of baseline (Figs. 6A, 6B; p=0.081 compared to time controls). Compared to male rats, the distribution of firing rates from G18 pregnant rats was significantly right-shifted (p<0.001) indicating a greater percentage of XII motoneurons from pregnant rats were resistant to allopregnanolone (Fig. 2C). In male rats, 50% of XII motoneurons were allopregnanolone-sensitive while only 23% of XII motoneurons from G18 pregnant rats were sensitive (Fig. 2D). Allopregnanolone-resistance in male rats was only 22% of XII motoneurons while 68% of XII motoneurons from G18 pregnant rats were resistant (Fig. 2D). Additionally, 94.2% (48 of 51) of allopregnanolone-resistant XII motoneurons decreased firing rates by >50% with muscimol application. Thus, allopregnanolone resistance in pregnant rat XII motoneurons was not due to local network effects.

XII motoneuron response to allopregnanolone occurs through $GABA_AR$

To test whether allopregnanolone sensitivity in male rats occurred through $GABA_AR$, slices from male rats (n=3 rats, 26 cells) were subfused with synaptic blockade aCSF solution, followed by bicuculline (100 μM) application for 20 min, before co-application with allopregnanolone (1.0 μM) for 45 min. XII motoneuron firing rates were at 147 ± 32% of baseline during allopregnanolone application (Figs. 7A, 7B; p<0.001 compared to allopregnanolone). Therefore, allopregnanolone appears to act as a positive allosteric modulator on $GABA_ARs$ to inhibit XII motoneurons in male rats.

Epsilon and delta subunit mRNA medullary regions

Epsilon subunit mRNA was quantified from tissue punches taken from medullary slices from male and G18 pregnant rats that included the following regions: XII motor nucleus, NTS, ventral respiratory column (VRC) region, pre-Bötzinger Complex (PBC) region, midline structures (MID), dorsolateral edges (LAT), and cortex (CTX) (Fig. 8A). Epsilon subunit mRNA expression in G18 pregnant and male rats were not different ($p=0.684$; Figs. 8B, 8C). However, in male and pregnant rats, epsilon subunit mRNA expression was increased in respiratory-related XII, NTS, VRC, and PBC regions compared to non-respiratory-related lateral medulla and cortical brain regions ($p\leq0.008$) These data suggest that respiratory-related nuclei have more epsilon mRNA than non-respiratory regions.

Delta subunit mRNA was quantified from the same tissue punches described above. Opposite to epsilon mRNA expression patterns, delta mRNA levels were significantly higher in the cortex compared to all medullary regions in male and pregnant rats ($p<0.001$; Figs. 9A, B). In G18 pregnant rats, delta mRNA levels were decreased in the XII ($p=0.017$), cortex ($p=0.007$), and lateral medulla ($p=0.003$) compared to male rats ($p<0.001$ for sex effect; Fig. 9A-D). Thus, delta subunit mRNA decreases in the XII motor nucleus and non-respiratory-related brain regions during pregnancy.

V. DISCUSSION

In acutely isolated medullary slices *in vitro*, XII motoneurons from G18 pregnant rats were more resistant to bath-applied allopregnanolone or pentobarbital compared to XII motoneurons from male and virgin female rats. Pentobarbital was used to pharmacologically test for epsilon subunit expression,

similar to previous studies from our group (Hengen *et al.*, 2009, 2011, 2012). This study, however, is the first to directly show that pregnant rat XII motoneurons are more resistant to allopregnanolone, the endogenous neurosteroid, under conditions of synaptic blockade. Delta subunit mRNA in the medulla decreased during pregnancy and may have contributed, in part, to the increased resistance to allopregnanolone. Taken together, these results suggest that epsilon subunit incorporation in $GABA_AR$ is increased during late pregnancy to protect adult XII motoneurons from excessive allopregnanolone-dependent inhibition.

$GABA_AR$ subunit reconfiguration during pregnancy

Pregnancy is an essential physiological process that induces widespread changes in $GABA_AR$ subunit expression. For example, delta subunits decrease in the hippocampus (Maguire and Mody, 2008), alpha-5 (Follesa *et al.*, 1998) and gamma-2 subunits (Follesa *et al.*, 2002) decrease in the cerebral cortex, and alpha-1 subunits increase in the hypothalamus (Concas *et al.*, 1999; Fenelon and Herbison, 1996; Follesa *et al.*, 1998). In addition, $GABA_AR$ sensitivity to drugs changes during pregnancy such that the efficacy of the $GABA_AR$ agonist (muscimol) increases and sensitivity to allopregnanolone is attenuated (Majewska *et al.*, 1989), which likely reflects pregnancy-induced changes in $GABA_AR$ subunit composition. Accordingly, there is increased epsilon subunit mRNA and immunoreactivity in the PBC region, and increased resistance to pentobarbital *in vivo* and *in vitro* (Hengen *et al.*, 2012).

Likewise, it is reasonable to speculate that several key elements of the respiratory control network in the brainstem need to be protected simultaneously from excessive allopregnanolone-dependent inhibition. Otherwise, there may be a critical loss of function that would be detrimental to the health of the pregnant female. Thus, one would predict that there will be increased epsilon

expression during pregnancy in respiratory rhythm-generating neurons, premotor neurons, motoneurons regulating the upper airway (such as XII motoneurons), and spinal motoneurons involved in pumping air. Consistent with this hypothesis, we show that pregnant rat XII motoneurons express $GABA_A$Rs (muscimol-sensitive) and increase their resistance to allopregnanolone *in vitro* while bathed with standard aCSF solution and synaptic blockade aCSF solution.

Epsilon subunit expression in pregnant rat XII motoneurons

Relatively little is known with regard to the specific physiological role of epsilon subunits in the CNS. In recombinant receptors, the epsilon subunit must combine with at least one alpha and one beta subunit to form a functional receptor with GABA-activated currents and ligand binding (Davies *et al.*, 1997; Whiting *et al.*, 1997). Inclusion of the epsilon subunit confers the following properties to $GABA_A$Rs: spontaneous opening, diminished outward rectification, insensitivity to benzodiazepines, slow receptor deactivation, altered receptor desensitization, altered sensitivity to positive modulation by anesthetics/neurosteroids and negative modulation by anabolic androgenic steroids (Davies *et al.*, 1997, 2001; Whiting *et al.*, 1997; Thompson *et al.*, 1998, 2002; Neelands *et al.*, 1999; Maksay *et al.*, 2003; Wagner *et al.*, 2005; Jones *et al.*, 2006). However, expression of these properties in native receptors is largely unknown.

Since $GABA_A$Rs containing epsilon subunits are found in cholinergic, dopaminergic, serotonergic, and noradrenergic neurons, it is postulated that epsilon subunits help regulate widespread neuromodulatory influences on brain function (Belujon *et al.*, 2009). In the brainstem, epsilon subunit mRNA is expressed in the raphe nuclei, A5 area, NTS, locus coeruleus, and dorsal vagal complex (Moragues *et al.*, 2000; Kasparov *et al.*, 2001). Recently, our group found that epsilon subunit expression

increased in cardiorespiratory-related brainstem regions during hibernation (Hengen *et al.*, 2009; 2011) and late pregnancy (Hengen *et al.*, 2012). The fundamental hypothesis that emerged from these studies is that epsilon subunit expression increases in brainstem cardiorespiratory neurons to protect against excessive positive allosteric modulation of $GABA_A$Rs caused by increased central levels of allopregnanolone. Furthermore, the threshold to increase epsilon subunit incorporation in $GABA_A$R appears to be lower in brainstem neurons compared to cortical neurons (Hengen *et al.*, 2009, 2011, 2012). These findings suggest that epsilon subunits play a key role in protecting and modulating cardiorespiratory function.

During late pregnancy, respiratory motor output on the phrenic nerve continued significantly longer following sequential pentobarbital injections in pregnant rats compared to non-pregnant female rats (Hengen *et al.*, 2012). It was noted that respiratory frequency was preserved rather than phrenic burst amplitude, suggesting the PBC neurons increased epsilon subunit incorporation in $GABA_A$Rs. Consistent with this hypothesis, neurons in the PBC-region of acutely isolated medullary slices from pregnant rats were more resistant to bath-applied pentobarbital compared to neurons from non-pregnant female and male rats (Hengen *et al.*, 2012). Also, epsilon subunit immunoreactivity was increased in the PBC-region in pregnant rats compared to non-pregnant female and male rats (Hengen *et al.*, 2012). However, it is unknown whether: 1) increasing epsilon subunit expression during pregnancy is unique to neurons in the PBC region or if other respiratory-related neurons, such as XII motoneurons, increase epsilon subunit expression; 2) increased epsilon expression also confers resistance to endogenous positive allosteric modulators, such as allopregnanolone, in medullary slices *in vitro*; and 3) increased resistance to pentobarbital or allopregnanolone is due to increased resistance in the recorded neuron or whether the resistance is due to the summation of drug effects on the local network.

The present study showed that that epsilon subunit incorporation in $GABA_AR$ increased during late pregnancy in XII motoneurons as evidenced by the increased resistance to both pentobarbital and allopregnanolone. The percentage of XII motoneurons that were resistant to pentobarbital application increased from 8% in male rats to 40% in G18 pregnant rats. Similarly, the percentage of allopregnanolone-resistant XII motoneurons increased from 22% in male rats to 68% in G18 pregnant rats. Thus, the percentage of resistant cells in pregnant rats increased 3-5 fold by gestational day 18. The increase in allopregnanolone resistance in G18 pregnant rat XII motoneurons appeared to be due to increased epsilon subunit incorporation in $GABA_ARs$ in the motoneuron itself because the resistance was observed under synaptic blockade conditions. Although XII motoneurons in medullary slices from pregnant rats were more resistant to pentobarbital in standard aCSF solution, these same neurons were unexpectedly sensitive to pentobarbital under synaptic blockade conditions. We hypothesize that pentobarbital sensitivity in XII motoneurons from pregnant rats in synaptic blockade solution was due to non-specific effects on neuronal excitability independent of $GABA_ARs$ or glycine receptors because bicuculline or strychnine did not block the pentobarbital-dependent inhibition (see below). Since only a percentage of respiratory motoneurons become resistant to pentobarbital or allopregnanolone, adjusting epsilon expression may be an important mechanism for fine tuning overall neuronal excitability within the XII motor nucleus.

The increased resistance of XII motoneurons in pregnant rats to pentobarbital and allopregnanolone is most consistent with an increase in epsilon subunit insertion into functional $GABA_ARs$. Epsilon subunit mRNA increases 4-fold in the entire medulla of pregnant rats compared to male and non-pregnant female rats (Hengen *et al.*, 2012), but the relative expression within specific medullary regions is not known. In this study, epsilon subunit mRNA was detected in all regions, but there was significantly higher expression in respiratory-related regions (*e.g.*, XII motor nucleus, PBC,

VRC, NTS) than other medullary regions (lateral trigeminal) or the cortex. Despite the increase in pentobarbital- and allopregnanolone-resistant XII motoneurons during pregnancy, there was no detectable change in XII motor nucleus epsilon subunit mRNA levels. This discrepancy may be due to: (1) decreased degradation of epsilon subunits during pregnancy, (2) increased trafficking of epsilon subunits to the plasma membrane during pregnancy, (3) increased activation of epsilon-containing $GABA_A$Rs by post-translational mechanisms (*e.g.*, phosphorylation or dephosphorylation), or (4) substrain differences (Sprague Dawley rats from Harlan *versus* Charles Rivers), (5) technical differences during tissue harvest (*e.g.*, rapid extraction of whole medulla versus taking punches from thick slices, and (6) other unknown mechanisms regulating function of epsilon-containing $GABA_A$Rs. Nonetheless, epsilon subunit mRNA was expressed in XII motor nuclei at levels higher than non-respiratory-related medullary regions and cortex. Taken together, our data provide evidence that epsilon subunits are expressed and functioning in the respiratory motor control system during pregnancy. XII motoneurons appear to actively compensate for increased inhibition during pregnancy to maintain appropriate excitability by becoming resistant to allopregnanolone. Thus, $GABA_A$R appear to reconfigure the subunits comprising the pentamer to induce resistance to positive allosteric modulation.

Caveats and limitations

One caveat is that the extracellular single-unit recordings in medullary brain slices were performed within the visible boundaries of the XII motor nuclei. It's possible that some of these recordings were performed on interneurons (rather and motoneurons) within the XII motor nucleus since no pharmacological or electrophysiological tests were used to positively identify XII motoneurons. Most of the neurons within the XII motor nucleus of rodents, however, are motoneurons rather than

interneurons because ~89% of neurons within murine XII motor nuclei are motoneurons (Sturrock *et al.*, 1991). Thus, our data likely reflect changes in XII motoneuron $GABA_A$Rs.

Our main experimental approach was to pharmacologically quantify epsilon subunit incorporation using resistance to positive allosteric modulators, such as pentobarbital and allopregnanolone. Pentobarbital was used extensively for this purpose by our group in previous publications (Hengen *et al.*, 2009, 2011, 2012), while this is the first study to use allopregnanolone to test for epsilon subunits. Thus, we tested for functional epsilon subunit expression rather than simply quantifying mRNA or protein. A caveat of this approach is that pentobarbital and allopregnanolone alter other neuronal properties in addition to potentiating $GABA_A$R function. For example, pentobarbital inhibits AMPA receptors in neonatal rat XII motoneurons (10-1000 μM; Essin *et al.*, 2002), serotonin 5-$HT_{3A/B}$ receptors (100-300 μM; Rusch *et al.*, 2007), and glycine receptors (1-3 mM, Mohammadi *et al.*, 2004). At the concentrations used in this study (200-300 μM), pentobarbital may have decreased XII motoneuron excitability by inhibiting AMPA receptors in addition to augmenting $GABA_A$R function when the slices were bathed in standard aCSF solution. However, $GABA_A$R blockade reversed the pentobarbital-dependent depression of PBC-region neuron spontaneous firing rates, suggesting that the pentobarbital effects were primarily via $GABA_A$Rs in the standard aCSF solution (Hengen *et al.*, 2012). In contrast, the pentobarbital-dependent decrease in XII motoneuron excitability in synaptic blockade solution was not blocked by simultaneous application of bicuculline and strychnine, which is consistent with pentobarbital acting non-specifically on other excitatory ion channels. Further studies are required to test this hypothesis directly.

Since allopregnanolone alters release of several neurotransmitters (*e.g.*, serotonin, norepinephrine, dopamine, glutamate, and glycine) in different parts of the brain, it's possible that allopregnanolone altered neurotransmitter release within the slice and thereby altered XII motoneuron

excitability. Likewise, allopregnanolone inhibits L-type calcium channels in the prefrontal cortex (Hu *et al.*, 2002; Zheng, 2009) and could have altered XII motoneuron excitability since newborn rat XII motoneurons express these channels (Viana *et al.*, 1993). However, bicuculline blocked the inhibitory effects of allopregnanolone in male rats in this study, suggesting that allopregnanolone acted primarily by augmenting $GABA_AR$ function rather than via non-specific effects on ion channels.

Finally, published data are conflicting on the ability of anesthetics (*i.e.*, pentobarbital) and anesthetic neurosteroids (*i.e.*, allopregnanolone) to potentiate epsilon-containing recombinant receptors (Davies *et al.*, 1997, 2001; Whiting *et al.*, 1997; Thompson *et al.*, 1998). This may be due to whether $GABA_AR$ subunit assemblies include: multiple epsilon subunits within a receptor or epsilon replacing alpha-1 at position 1, beta-2 at position 4 or gamma-2 at position 5 (Jones *et al.*, 2007; Bollan *et al.*, 2008). Co-assembly with various subunits may contribute to functional variability within $GABA_AR$ containing epsilon subunits (Jones *et al.*, 2007). For example, changing $GABA_AR$ subunit composition alters allosteric modulation properties (Olsen, 1998) channel gating (Gingrich *et al.*, 1995; Lavoie *et al.*, 1997), formation of GABA binding sites (Kash *et al.*, 2003), and current rectification (Davies *et al.*, 1997, 2001). With respect to native $GABA_ARs$, epsilon subunits appear to confer insensitivity to a variety of positive allosteric modulators including pentobarbital (Hengen *et al.*, 2009, 2012), allopregnanolone, and ethanol (Hengen *et al.*, 2011). The subunit co-assembly patterns and expression of other characteristics attributed to epsilon subunits remain to be determined in future studies.

Decreased delta subunit expression does not fully explain resistance to positive allosteric modulation

Delta subunits confer distinct properties to $GABA_ARs$ that are in sharp contrast to the characteristics attributed to epsilon subunit inclusion. $GABA_AR$ containing delta subunits are found in

peri- or extrasynaptic locations and mediate a GABA-dependent tonic current that decreases neuronal excitability (Wei *et al.*, 2003; Glykys and Mody, 2006). Delta-containing $GABA_A$Rs are highly sensitive to potentiation by positive allosteric modulators, such as neurosteroids (Wohlfarth *et al.*, 2002). For example, mice lacking the delta subunit are markedly less sensitive to neurosteroids *in vivo* (Mihalek *et al.*, 1999) and *in vitro* (Spigelman *et al.*, 2003). During pregnancy, delta subunit expression and tonic inhibition are decreased in hippocampal neurons (Maguire and Mody, 2008). Further, delta subunits appear to be expressed in XII motoneurons from P3-P15 mice because exogenous GABA or THIP (delta subunit specific agonist) application induces a tonic inhibitory current (Numata *et al.*, 2012). Based on these findings, it's possible that decreased delta subunit expression in pregnant rat XII motoneurons contributed to the decreased allopregnanolone sensitivity we observed. Consistent with this hypothesis, we found a nearly 10-fold decrease in delta subunit mRNA in XII motor nuclei from pregnant rats compared to male rats. Two factors, however, suggest that potential changes in delta subunit expression in pregnant rat XII motoneurons are not likely to fully explain our results. First, application of THIP to male rat XII motoneurons at a concentration considered "specific" for delta subunit-containing $GABA_A$Rs (100 nM; Meera *et al.*, 2011) did not decrease XII motoneuron spontaneous firing rates (Johnson Lab, unpublished observations). This suggests that delta subunit expression in male rats did not contribute to the sensitivity of XII motoneurons to allopregnanolone in the first place. Thus, decreasing delta subunit expression in pregnant rats could not account for the allopregnanolone resistance if a delta subunit-dependent effect could not be demonstrated in male rat XII motoneurons. Second, even if there were no delta subunits expressed in XII motoneurons, the extensively expressed conventional $GABA_A$Rs (*e.g.*, composed of alpha-1, beta-2, gamma-2 subunits) would still be sensitive to positive allosteric modulation by allopregnanolone. Modulation of delta subunits could contribute to the relative sensitivity of $GABA_A$Rs to allopregnanolone, but a lack of delta subunits cannot explain allopregnanolone

resistance. Instead, increased insertion of functional epsilon subunits into $GABA_A$Rs is the best explanation for allopregnanolone resistance.

Potential clinical significance

Modulation of XII motoneuron excitability is important because low excitatory drive to tongue muscles is hypothesized to contribute to sleep-disordered breathing, such as obstructive sleep apnea (reviewed in Horner, 2009). Although somewhat controversial, there is evidence suggesting that sleep-disordered breathing increases during pregnancy in women and ranges from mild disease (habitual snoring in 14-45% of pregnant women) to frequent apneic events with an underreported prevalence (reviewed in Bourjeily et al, 2010; Venkata and Vekatashiah, 2009). In addition, sleep-disordered breathing is associated with substantial maternal morbidity (gestational hypertension, diabetes, preeclampsia, unplanned cesarean delivery) and fetal morbidity (preterm delivery, fetal growth restriction and respiratory acidosis) (Venkata and Vekatashiah, 2009; Bourjeily *et al.*, 2010, Izci-Balserak and Pien, 2010, Louis *et al.*, 2010). While pregnancy-induced sleep-disordered breathing is thought to have a neural component (Maasilta *et al.*, 2001; Olivarez *et al.*, 2010), the sleep-related neural mechanisms contributing to upper airway obstruction in pregnant women are poorly understood. It's possible that a portion of pregnant women may have insufficient epsilon subunit insertion into $GABA_A$RS in XII motoneurons so that upper airway patency during sleep is compromised due to increased inhibition of XII motoneurons by the combination of increased central allopregnanolone levels and sleep-related withdrawal of excitatory synaptic inputs.

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Table 1. Sample number for each rat group in mRNA quantification studies.

Rat Type	mRNA type	CTX	XII	NTS	VRC	PBC	MID	LAT
Male	epsilon	9	9	8	9	8	8	8
Pregnant	epsilon	5	3	5	7	7	5	5
Male	delta	10	10	9	10	9	10	12
Pregnant	delta	5	5	5	6	6	7	7

Figure 1: Extracellular recording sites. Multichannel electrode arrays were positioned in the XII nucleus such that array tips spanned the nucleus from lateral edge to slice midline. Areas of electrode placements are shaded in gray.

Fig. 1

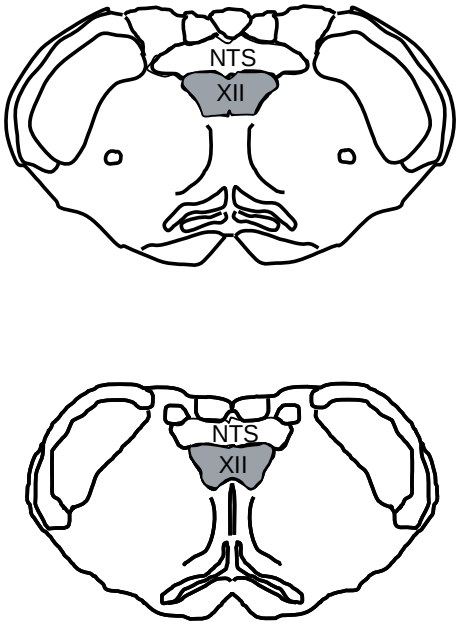


Figure 2: XII motoneurons from pregnant rats have increased resistance to pentobarbital. (A) Sequential pentobarbital applications (200 μM, 300 μM; 45 min each) decreased spontaneous XII motoneurons firing rates in male (white circles) and female (gray triangles) rats compared to time controls (small black circles) and pregnant rats (black squares). XII motoneurons from all groups were sensitive to muscimol application (at 120-135 min). (B) XII motoneurons from pregnant rats (black bars) were more resistant to pentobarbital compared to male (white bars) and female (gray bars) rats ($p<0.05$). (C) The cumulative histogram shows that compared to male (dashed line) and female (dotted line) rats the curve for pregnant rats (black line) was shifted to the right, indicating that a greater portion of XII motoneurons were resistant to pentobarbital. (D) Categorizing XII motoneuron average firing rates during pentobarbital application (300 μM) into sensitive (<40% of baseline; white), intermediate (40-80% of baseline; gray) or resistant (>80% of baseline; black) groups showed that in male rats only 8% of neurons were resistant to pentobarbital whereas 40% of neurons from pregnant rats were resistant. (Norml. is an abbreviation for normalized) *indicates $p<0.05$ compared to other drug application groups; # indicates $p<0.05$ compared to time controls; † indicates drug effect, $p<0.05$.

Fig. 2

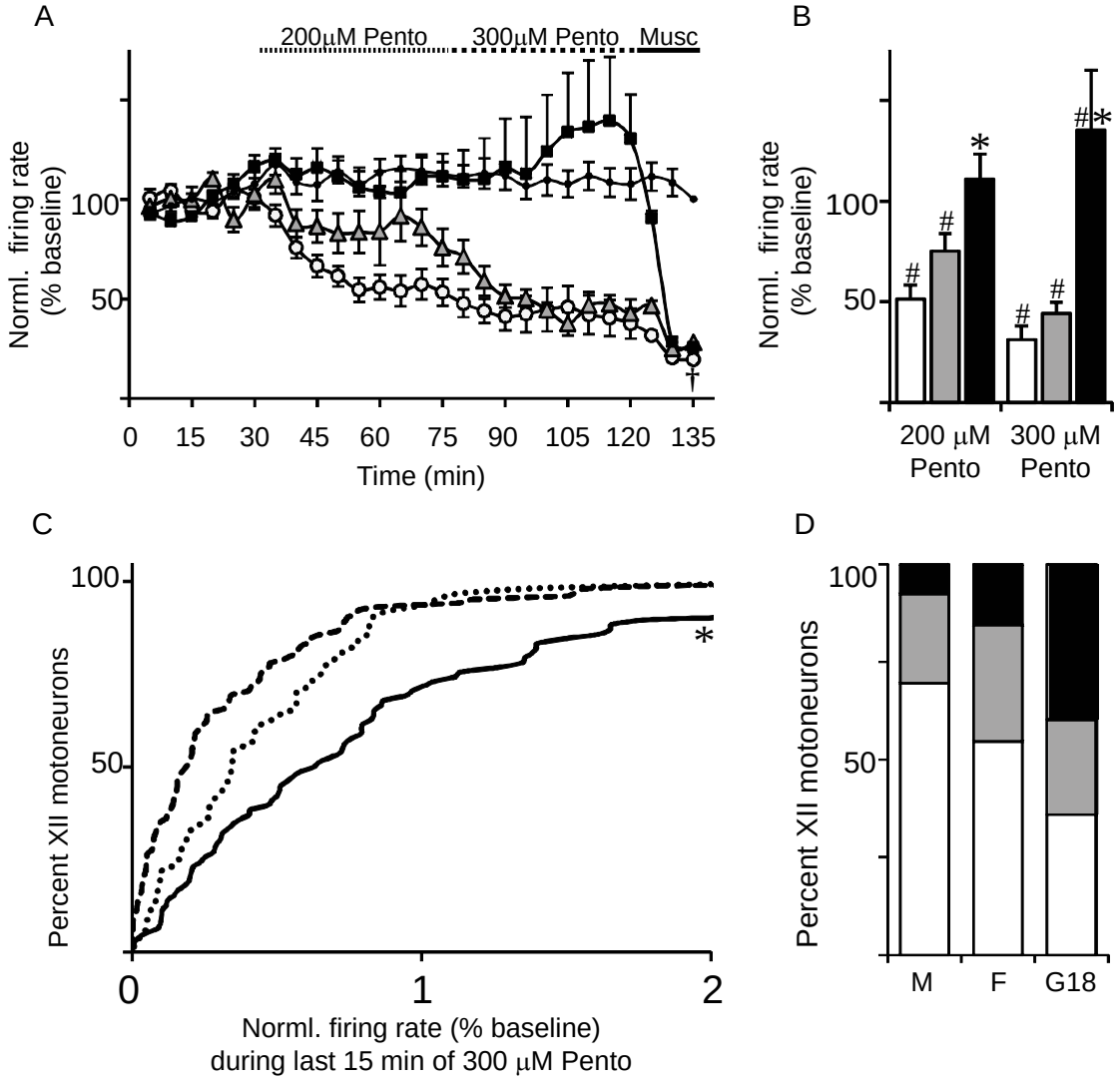

A
200μM Pento
300μM Pento
Musc
Norml. firing rate (% baseline)
100
50
0 15 30 45 60 75 90 105 120 135
Time (min)
†
B
Norml. firing rate (% baseline)
100
50
#
*
#*
200 μM Pento
300 μM Pento
C
Percent XII motoneurons
100
50
0 1 2
Norml. firing rate (% baseline) during last 15 min of 300 μM Pento
D
Percent XII motoneurons
100
50
M
F
G18

Figure 3: Pentobarbital sensitivity under synaptic blockade conditions. (A) While bathed in synaptic blockade aCSF solution, bath-applied pentobarbital (300 μM) decreased XII motoneuron firing rates in male (white circles) and pregnant rats (black squares) rats compared to time controls (small black circles). XII motoneurons from all groups were sensitive to muscimol application. (B) Pentobarbital application decreased average firing rates in XII motoneurons from male (white bar) and pregnant (black bar) rats to 52-58% of baseline. (C) Cumulative histogram showing male (dashed line) and pregnant rats (black line) had similar distributions of cells, indicating a similar resistance to pentobarbital. (D) XII motoneurons were categorized based on their average firing rates during 300 μM pentobarbital application as either sensitive (<40% of baseline; white), intermediate (40-80% of baseline; gray) or resistant (>80% of baseline; black). In slices from male and pregnant rats, only 25% of cells were resistant to pentobarbital. Symbols are as in Fig. 2.

Fig. 3

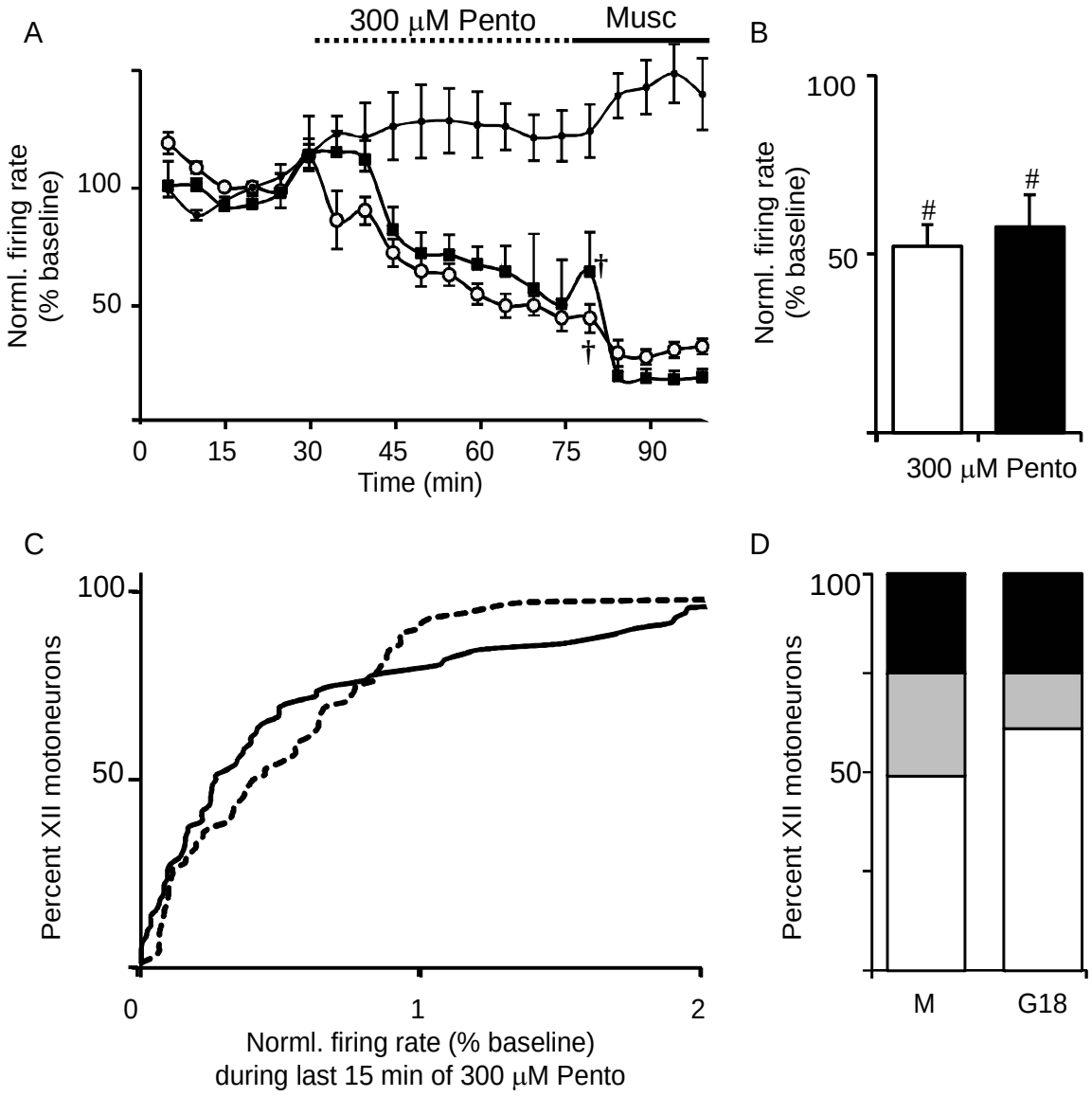

A
300 μM Pento
Musc
Norml. firing rate
(% baseline)
100
50
0
15
30
45
60
75
90
Time (min)
†
†
B
100
50
Norml. firing rate
(% baseline)
#
#
300 μM Pento
C
100
50
Percent XII motoneurons
0
1
2
Norml. firing rate (% baseline)
during last 15 min of 300 μM Pento
D
100
50
Percent XII motoneurons
M
G18

Figure 4: Pentobarbital sensitivity is not mediated through $GABA_A$ or glycine receptors in XII motoneurons from pregnant rats. (A) To test whether pentobarbital sensitivity under conditions of synaptic blockade in XII motoneurons from pregnant rats was mediated through $GABA_A$Rs, bicuculline (100 μM, BIC) was applied 20 min prior to and during pentobarbital (300 μM) application. XII motoneurons firing rates decreased with bicuculline application similar to when only pentobarbital was applied (see Fig. 2). To test whether pentobarbital sensitivity occurred through GABAARs or glycine receptors (major inhibitory chloride channels), bicuculline (100 μM) and strychnine (5 μM, STRYCH) were applied prior to and during pentobarbital (300 μM) co-application. Spontaneous XII motoneurons firing rates decreased with BIC-STRYCH application similarly to when only pentobarbital was applied. (B) Average firing rates in XII motoneurons from pregnant rats decreased with pentobarbital (white bar), BIC + pentobarbital (gray bar), and BIC + STRYCH + pentobarbital (black bar) to ~59% of baseline. Time control data are shown with small solid circles. Symbols are as in Fig. 2.

Fig. 4

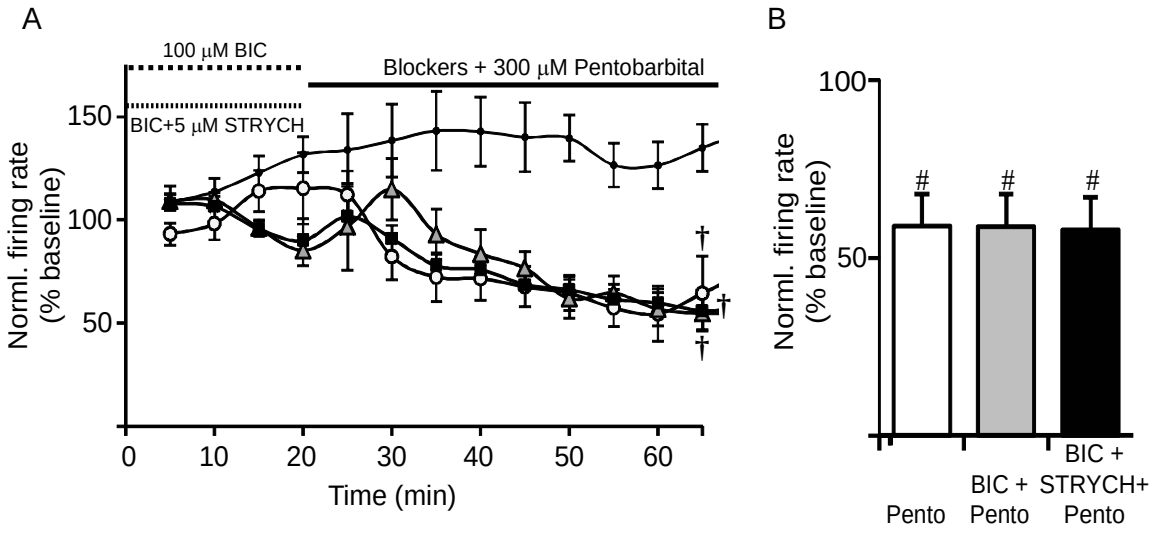

A
100 μM BIC
Blockers + 300 μM Pentobarbital
BIC+5 μM STRYCH
Norml. firing rate
(% baseline)
150
100
50
0
10
20
30
40
50
60
Time (min)
†
B
100
50
Norml. firing rate
(% baseline)
#
#
#
Pento
BIC +
Pento
BIC +
STRYCH+
Pento

Figure 5: XII motoneurons from pregnant rats have increased resistance to allopregnanolone. (A) Allopregnanolone (1.0 μM) application decreased spontaneous XII motoneuron firing rates in male rats (white circles) compared to pregnant rats (black squares) and time controls (small black circles). (B) Allopregnanolone application decreased average firing rates in XII motoneurons from male rats (white bar) while XII motoneuron firing rates are maintained at or above baseline values in in pregnant rats (black bar). (C) The cumulative histogram shows that the distribution of neuronal firings rates in pregnant rats (black line) was right-shifted compared to male (dashed line) rats, indicating that a greater portion of XII motoneurons from pregnant rats were resistant to allopregnanolone. (D) Categorizing XII motoneuron average firing rates into sensitive (<40% of baseline; white), intermediate (40-80% of baseline; gray) or resistant (>80% of baseline; black) groups showed that in male rats only 40% of cells were resistant to pentobarbital whereas 68% of cells from pregnant rats were resistant. Symbols are as in Fig. 2.

Fig. 5

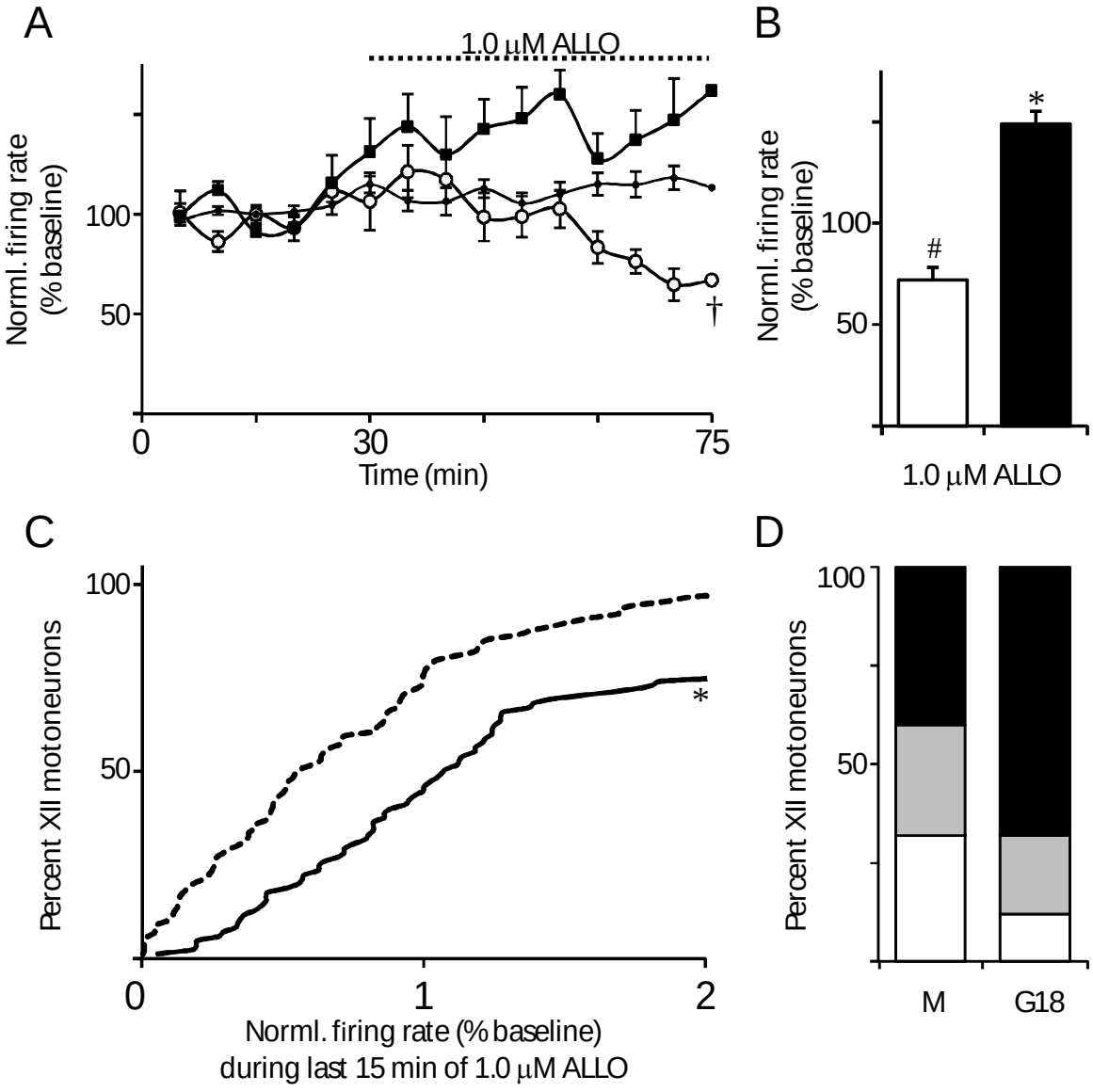

A
1.0 μM ALLO
Norml. firing rate
(% baseline)
100
50
0
30
75
Time (min)
†
B
Norml. firing rate
(% baseline)
100
50
#
*
1.0 μM ALLO
C
Percent XII motoneurons
100
50
0
1
2
*
Norml. firing rate (% baseline)
during last 15 min of 1.0 μM ALLO
D
Percent XII motoneurons
100
50
M
G18

Figure 6: XII motoneuron response to allopregnanolone is intrinsic to motoneurons. (A) Under conditions of synaptic blockade, allopregnanolone (1.0 μM) decreased XII motoneurons firing rates in male rats (white circles) compared to pregnant rats (black squares) and time controls (small black circles). (B) Average firing rates in XII motoneurons from male rats (white bar) decreased to 54 ± 7% of baseline, while XII motoneurons from pregnant rats (black bar) increased to 146 ± 17% of baseline during allopregnanolone application. (C) The cumulative histogram shows that the distribution of XII motoneuron average firing rate during allopregnanolone application was right-shifted in pregnant rats (black line) compared to male (dashed line) rats. (D) XII motoneuron average firing rates were categorized into sensitive (<40% of baseline; white), intermediate (40-80% of baseline; gray) or resistant (>80% of baseline; black) groups to show in male rats only 22% of cells were resistant to allopregnanolone whereas 68% of cells from pregnant rats were resistant. Symbols are as in Fig. 2.

Fig. 6

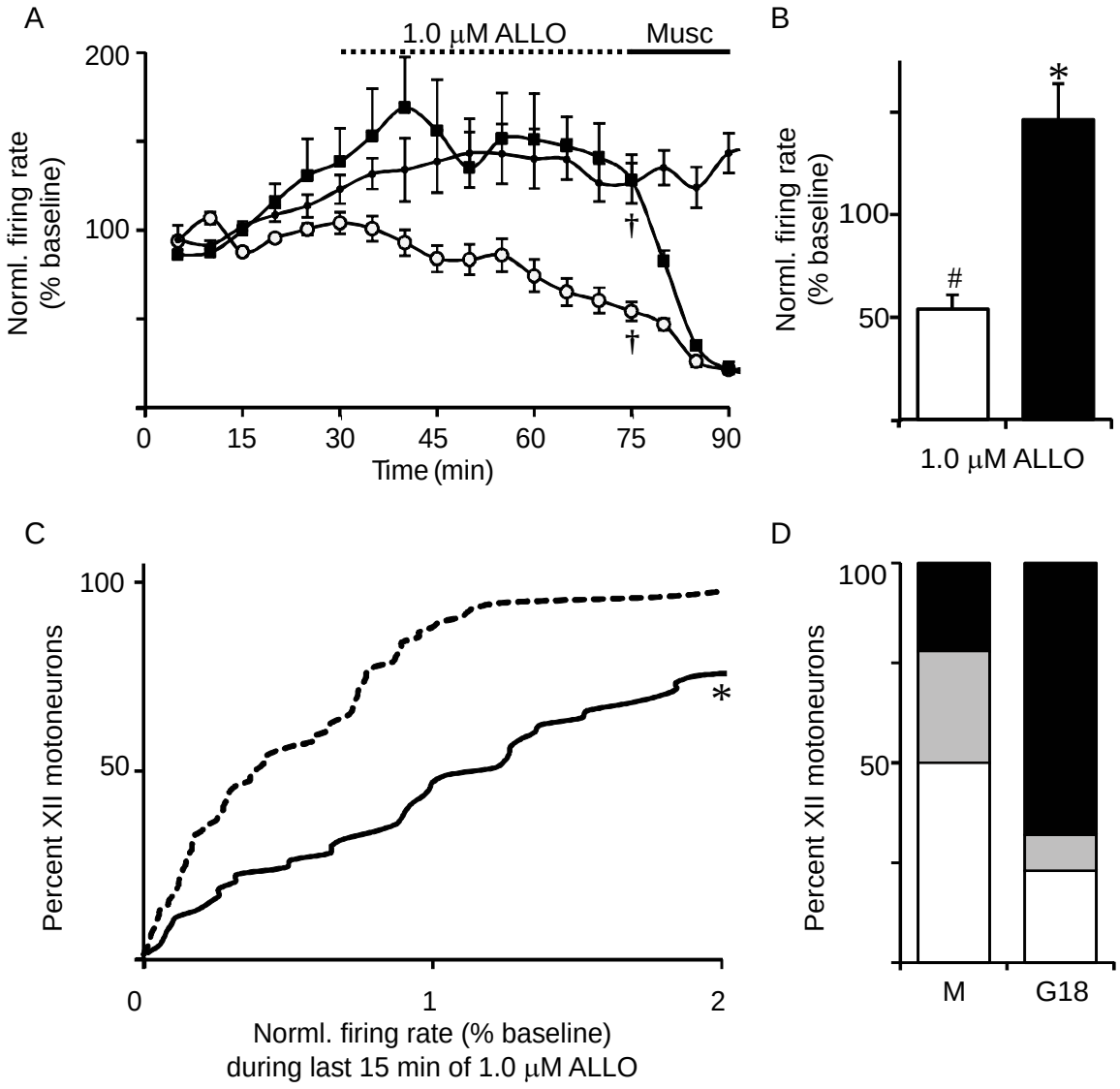

A
1.0 μM ALLO
Musc
200
100
Norml. firing rate
(% baseline)
0
15
30
45
60
75
90
Time (min)
†
†
B
Norml. firing rate
(% baseline)
100
50
#
*
1.0 μM ALLO
C
100
50
Percent XII motoneurons
0
1
2
Norml. firing rate (% baseline)
during last 15 min of 1.0 μM ALLO
*
D
100
50
Percent XII motoneurons
M
G18

Figure 7: XII motoneuron response to allopregnanolone is mediated by $GABA_ARs$. (A) In synaptic blockade aCSF solution, bicuculline (100 μM, BIC) was applied 20 min prior to and during allopregnanolone application (1.0 μM). While allopregnanolone application alone (white circles) decreased spontaneous XII motoneuron firing rates, co-application of bicuculline (black squares) blocked the allopregnanolone-dependent decrease in firing rates. (B) Average firing rates in XII motoneurons decreased to 54 ± 7% of baseline in slices from male rats with allopregnanolone application alone (white bar). Co-application of bicuculline blocked the allopregnanolone-dependent decrease in average firing rates (black bar). Symbols are as in Fig. 2.

Fig. 7

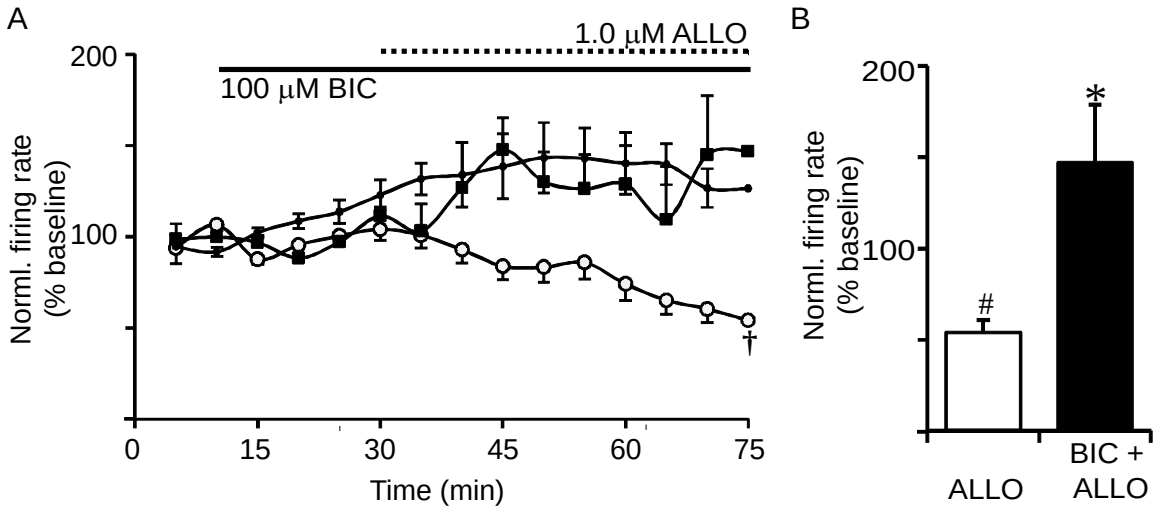

A
1.0 μM ALLO
100 μM BIC
200
100
Norml. firing rate
(% baseline)
0
15
30
45
60
75
Time (min)
†
B
200
100
Norml. firing rate
(% baseline)
#
*
ALLO
BIC +
ALLO

Figure 8: Epsilon subunit mRNA expression in male and pregnant rats. (A) Regions of slices collected for RT-qPCR analysis. (A, B) Epsilon subunit mRNA expression was increased in respiratory-related nuclei compared to the cortex in pregnant (B) and male (C) rats. * indicates $p<0.05$ compared to cortex within the rat group.

Fig. 8

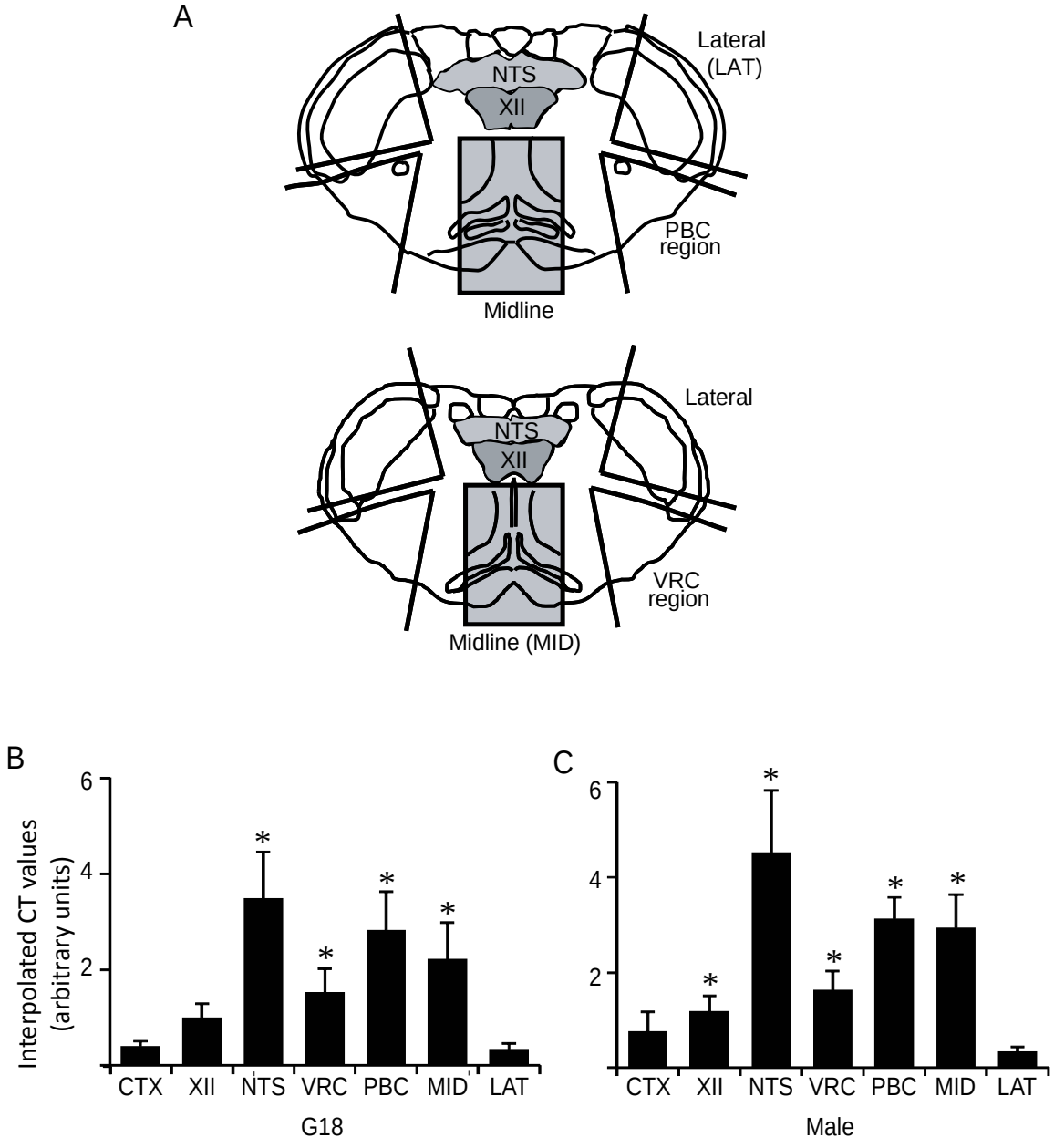
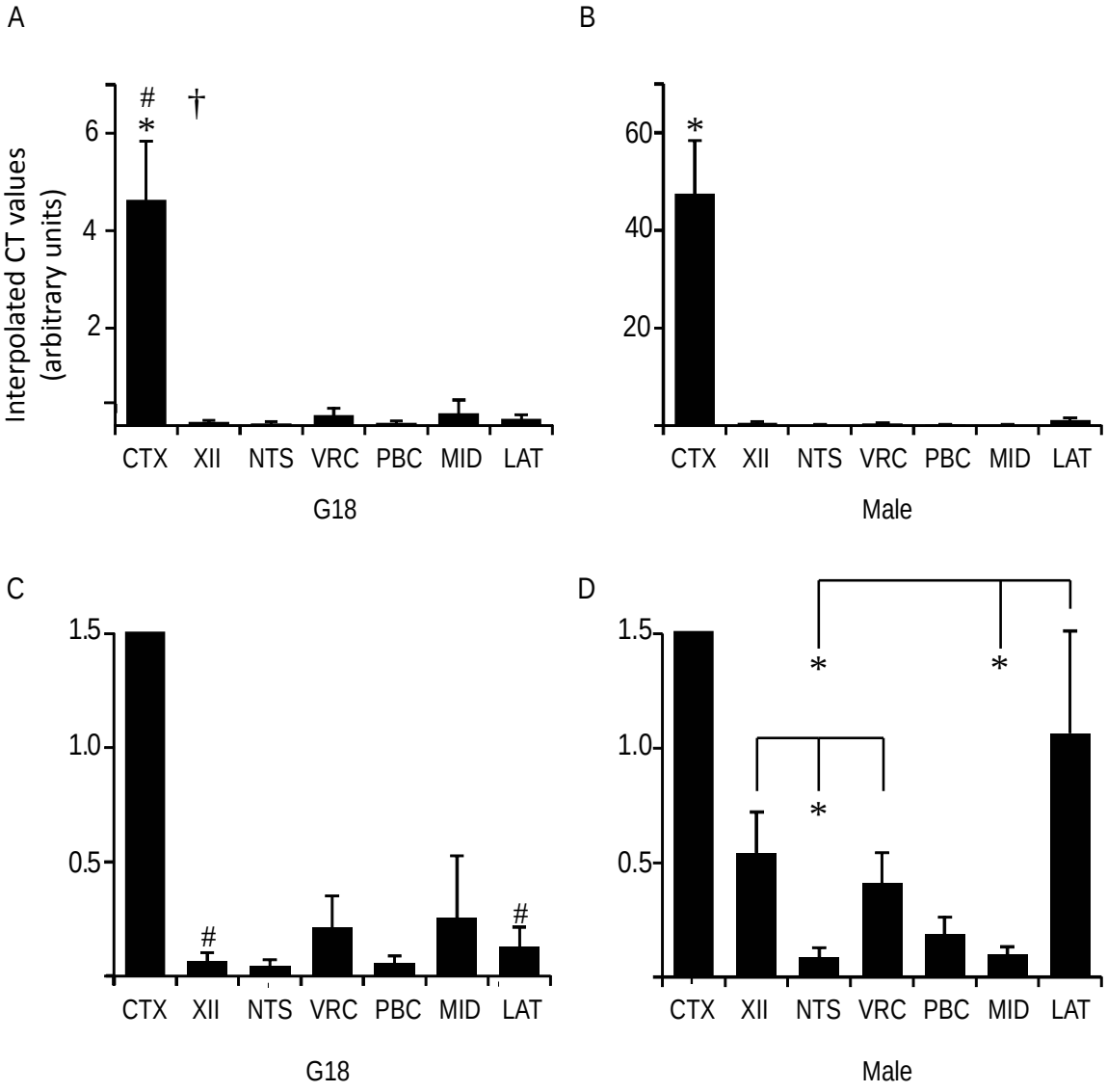

A
Lateral
(LAT)
NTS
XII
PBC
region
Midline
Lateral
NTS
XII
VRC
region
Midline (MID)
B
Interpolated CT values
(arbitrary units)
6
4
2
CTX
XII
NTS
VRC
PBC
MID
LAT
G18
C
6
4
2
CTX
XII
NTS
VRC
PBC
MID
LAT
Male

Figure 9: Delta subunit mRNA expression is decreased in pregnant rats compared to males. (A, B) Pregnant rats (A) had lower cortical delta subunit mRNA expression compared to male rats (B). Cortical delta subunit mRNA expression was significantly higher than medullary levels in pregnant (A) and male (B) rats. (C, D) Same data as in A & B with a smaller y-axis scale to better visualize variability in medullary delta subunit mRNA expression. Pregnant rats (C) had lower XII and dorsolateral (LAT) mRNA expression compared to male rats (D). *indicates regional differences within a rat group $p<0.05$, # indicates pairwise differences between male and pregnant rats $p<0.05$, and † indicates $p<0.0001$ for male vs. pregnant effect.

Fig. 9


A
B
C
D
Interpolated CT values
(arbitrary units)
6
4
2
60
40
20
1.5
1.0
0.5
CTX
XII
NTS
VRC
PBC
MID
LAT
G18
Male
#
*
†

Chapter 3:

Abrupt changes in pentobarbital sensitivity in respiratory-related brain regions during the transitional period in respiratory control development.

Sara M.F. Turner, Stephen M. Johnson

I. ABSTRACT

During postnatal development in the rat medulla, there is a transitional period from P11-P14 when neurotransmitters change concentration, and receptor subunit composition shifts towards the mature phenotype. During this time, brain concentrations of the neurosteroid, allopregnanolone, increase by 3-4 fold. Allopregnanolone increases chloride ion influx in $GABA_A$ receptors ($GABA_A$Rs), thereby enhancing neuronal inhibition. Since $GABA_A$Rs in medullary neurons regulate respiratory rhythm generation, upper airway patency, and sensory integration, excessive allopregnanolone-dependent inhibition could disrupt blood-gas homeostasis. Inserting epsilon subunits into $GABA_A$Rs confers resistance to positive allosteric modulators (allopregnanolone, pentobarbital). We hypothesized that epsilon subunit incorporation in $GABA_A$Rs will increase in respiratory-related brainstem regions to protect against increased allopregnanolone concentrations during P11-P14. To address this question, medullary and cortical slices were taken from P10-P20 rats. Extracellular silicon multichannel electrodes recorded spontaneous action potentials in pre-Botzinger Complex (PBC)-region, hypoglossal (XII) motor nucleus, nucleus tractus solitariius (NTS) neurons, and cortex. To pharmacologically test for epsilon subunit incorporation in $GABA_A$R, slices were sequentially exposed to 200 and 300 μM pentobarbital (45 min each). Neurons firing at >80% of baseline firing rates were considered pentobarbital-resistant and likely expressing epsilon subunits. During the P10-P15 period, pentobarbital resistance increased abruptly for one day in PBC-region (P14) and NTS (P13) neurons ($p<0.05$). In contrast, pentobarbital resistance in XII motoneurons decreased from P11-P15 with the greatest pentobarbital sensitivity at P14 ($p<0.05$). For cortical neurons, pentobarbital resistance was lower compared to medullary regions and there was a modest abrupt decrease at P12 ($p<0.05$). These data suggest that medullary epsilon subunit expression changes abruptly in an age- and region-specific manner and is not directly correlated with central allopregnanolone levels.

II. INTRODUCTION

GABAergic synaptic transmission plays a key role in the respiratory control network and $GABA_A$Rs are widely expressed on respiratory neurons. $GABA_A$Rs are responsible for fast, synaptic inhibition as well as modulating neuronal excitability with a tonic inward inhibitory current. For example, in PBC neurons, $GABA_A$Rs modulate neuronal excitability and regulate respiratory rhythm generation and pattern (Shao and Feldman, 1997). Also, tonic GABAergic input constrains the firing rate of medullary respiratory neurons by 35-50% (Zuperku and McCrimmon, 2002). Excessive or insufficient GABAergic signaling significantly disrupts respiratory output (Koshiya and Guyenet, 1996; Paton and Richter, 1995). $GABA_A$Rs are considered pentameric (Bonnert *et al.*, 1999) and typically composed of two alpha subunits, two beta subunits, and one gamma subunit. Inclusion of other subunits (delta, epsilon, theta, pi or rho) modulates GABA affinity, efficacy, channel gating properties (Gringrich *et al.*, 1995; Lavoie *et al.*, 1997; Kash *et al.*, 2003), and sensitivity to positive allosteric modulators (enhance chloride ion influx; Olsen, 1998). The variety of $GABA_A$R functions is attributable to the subunit composition of the receptor, which changes during development. However, the roles of specific $GABA_A$R subtypes during respiratory control development are poorly understood.

There is a transitional period in respiratory control development during postnatal days P11-P14 that is characterized by abrupt, dramatic changes in receptor subunit composition, and neurotransmitter concentrations in the PBC (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). This transitional period is referred to as a “critical period” by Wong-Riley and colleagues, although definitive data showing that deprivation of sensory inputs during this period causes long-lasting changes into adulthood (*i.e.*, according to Hubel and Wiesel’s classical definition of critical period) have not been published to date. To be consistent with the major research group that has

investigated this period, we will refer to postnatal days 11-14 as the critical period in respiratory control from this point forward. On postnatal day 12 in PBC and NTS neurons, $GABA_AR$ subunit composition switches from containing predominately alpha-3 subunits to alpha-1 subunits (Liu and Wong-Riley, 2004; 2006), which likely improves the efficacy of GABAergic inhibition (Wong-Riley *et al.*, 2013). Likewise, in the XII motor nucleus, GABAergic inhibition is increased and excitatory signaling is decreased in P12-13 rats (Gao *et al.*, 2011). Further, in all three regions, glutamate and NMDAR1 decrease, whereas GABA, $GABA_BR$, glycine receptors, and GluR2 increase at P12 (Liu and Wong-Riley, 2005). Overall, these changes may contribute to decreased neuronal excitability and potentially result in a blunted hypoxic and hypercapnic ventilatory responses from P12-P16 (Liu *et al.*, 2006, 2009; Wong-Riley and Liu, 2008; Holley *et al.*, 2012). The compensatory mechanisms within the respiratory control system to protect breathing during this critical period in development are not known. It remains unclear whether $GABA_AR$ change subtypes to stabilize respiratory neuron output during this time.

Recently, our group has identified a novel compensatory mechanism within the respiratory control system: incorporation of the epsilon subunit into $GABA_ARs$ to confer insensitivity to positive allosteric modulators (Hengen *et al.*, 2012; Turner *et al.*, in preparation). Allopregnanolone is an inhibitory neurosteroid that acts as a positive allosteric modulator at $GABA_ARs$. When central allopregnanolone concentrations increase up to 3-fold during pregnancy in rats (Concas *et al.*, 1998), epsilon subunit incorporation in $GABA_ARs$ increases in PBC-region neurons (Hengen *et al.*, 2012) and XII motoneurons (Turner *et al.*, in preparation). Interestingly, cortical allopregnanolone concentrations also increase 3-4 fold in P10 and P14 rats, which is during the critical period in respiratory control development (Grobin and Morrow, 2001). However, it is not known whether medullary allopregnanolone concentrations and epsilon subunit expression are correlated in respiratory-related medullary regions during the critical postnatal developmental period. Based on our previous work, we

hypothesize that epsilon subunits are expressed in respiratory-related medullary regions and increase due to increased medullary allopregnanolone levels during the critical postnatal developmental period.

To address these questions, we recorded spontaneous action potentials in PBC-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 functional epsilon subunit expression, medullary and cortical slices were exposed to pentobarbital and the neuronal response was quantified as the percent change from baseline firing rate. Pentobarbital-resistant neurons did not decrease their spontaneous action potential firing rate by more than 20% during drug application and were considered as putatively expressing epsilon subunits. Our results show that there were abrupt age- and region-dependent changes in medullary epsilon incorporation that were not correlated with allopregnanolone levels during the critical postnatal developmental period.

III. METHODS

Electrophysiological brain slice recordings in vitro

Experimental procedure: 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 animals were used for brain slice electrophysiology (see Tables 1 and 2 for numbers of animals used for each experiment). Brains were removed, coronal medullary and cortical slices (400 µm thick) were cut in ice-cold standard artificial cerebrospinal fluid (aCSF) solution with a vibrating

microtome (Campden Instruments, Layfayette, IN, USA). Cortical slices contained primary motor and somatosensory areas. Medullas were isolated and removed by making transverse cuts at spinal segment C1 and the pontomedullary junction. A series of 3-4 medullary slices used for recording contained the XII motor nucleus, NTS, and the rostral ventrolateral medulla containing the compact nucleus ambiguus (hereafter referred to as the PBC-region) (Fig. 1). Slices were immediately placed into an interface recording chamber (Warner Instruments, Hamden, CT, USA) and subfused with warm standard aCSF solution (8 ml/min). Slices were maintained at 37°C by an automated temperature controller (Harvard Apparatus, Holliston, MA, USA). Spontaneous neuronal activity was recorded from PBC-region, XII, 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, each with four recording sites. 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.

Experimental Protocol: Slices were allowed to equilibrate in standard aCSF solution at 37°C with electrodes inserted for 60-90 min before recording baseline activity for 30 min. The standard aCSF solution composition was (in mM): 120 NaCl, 26 $NaHCO_3$, 20 glucose, 2.0 $MgSO_4$, 1.0 $CaCl_2$, 1.25 Na_2HPO_4, 7.0 KCl (KCl was elevated to increase yield of neuronal recordings). Following equilibration and baseline recordings, 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. During the last 30 min of some experiments, muscimol (20 µM, $GABA_A$ agonist, Tocris Bioscience, Ellisville, MO, USA) was bath-applied to the slices to confirm the presence of functional $GABA_A$Rs (Table 3).

Electrophysiological data analysis

Raw extracellular recordings of spontaneous action potentials were processed as described previously (Hengen *et al.*, 2009). Individual neurons were identified using Principal Component Analysis (Adamos *et al.*, 2008). 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. Waveforms recorded on multiple, adjacent channels were counted only once. Neuronal waveforms were discarded from analysis if any one of the following criteria were met: mean baseline firing rate was <0.01 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 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 min). Based on these criteria, 9.3% of waveforms and 0.1% of data bins were discarded.

Statistical analysis

Normalized, average firing rates from the last 15 min of drug application were used for statistical analysis. To test for significant differences between ages or regions over the range of data from P10-P20, 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). 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.

IV. RESULTS

Time control experiments: spontaneous neuron firing rates were constant for >3h in vitro

To confirm that spontaneous activity neuronal remained unaltered for the duration of our experiments, slices from P10-P15 and P20 rats were subfused for 4 h in standard aCSF solution (Table 1). Neurons from all brain regions produced spontaneous action potentials for the duration of the experiment. Data from each brain region were pooled across ages from P10-P15 because all groups produced unaltered activity ($P>0.05$; Fig. 2A). At the end of time control experiments, average neuronal firing rates were 124±8% of baseline in PBC-region neurons, 119±8% of baseline in XII motoneurons, 113±6% of baseline in NTS neurons and 107±3% of baseline in cortical neurons. Since P20 is outside of the critical developmental period, data from P20 rats were not pooled. Similar to the critical period time control data, average neuronal firing rates were 96±17% of baseline in PBC-region neurons, 115±12% of baseline in XII motoneurons, 122±9% of baseline in NTS neurons and 119±11% of baseline in cortical neurons at the end of time control experiments. There were no differences between P10-P15 and P20 time control data ($P>0.05$). The GABA agonist muscimol (20 μM) was applied during the final 15 min of the time control experiments to test whether activating $GABA_AR$ decreases spontaneous neuronal activity. Muscimol application decreased average firing rates by >40% of baseline values in 265 of 270 neurons.

Pentobarbital application decreases spontaneous action potential firing rates

Generally, pentobarbital bath application (200 μM and 300 μM) was sufficient to decrease average neuronal spontaneous firing rates by >50% of normalized baseline values (Fig. 2B). However, during the critical period, specific brain regions on particular days increased or decreased pentobarbital sensitivity (Fig. 2B, black squares), consistent with changes in $GABA_AR$ subunit composition. During muscimol application, average neuronal firing rates decreased to only 22-29% of baseline (Fig. 2B). From all brain regions, across all age groups, only 2% of neurons (15 out of 701) were pentobarbital- and muscimol-resistant (Table 3).

Pentobarbital resistance abruptly increased in PBC-region neurons at P14

In PBC-region neurons, pentobarbital application decreased spontaneous firing rates similarly across ages except at P14 when an increase in pentobarbital resistance was noted. During the last 15 min of the 200 μM pentobarbital application, the average PBC-region neuron firing rate was 61-73% of baseline in P10-P13 rats, increased to 133 ± 22% of baseline in P14 rats, and then returned to 63-69% of baseline in P15 and P20 rats ($p<0.05$ at P14; $p=0.002$ for age effect; Figs. 2B, 3A). Abrupt changes occurred in pentobarbital sensitivity from P13 to P14 and from P14 to P15 (Fig. 3A). Since pentobarbital-resistant neurons were a subpopulation, average firing rates from the last 15 min of the 200 μM pentobarbital application were classified as sensitive (<40% of baseline), intermediate (40-80% of baseline) or resistant (>80% of baseline). The percentage of resistant PBC-region neurons increased from 16% at P11 to 39% at P13 and peaked at 47% at P14, before decreasing to 32-35% at P15 and P20 (Fig. 3B). Pentobarbital-sensitive PBC-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 μM pentobarbital, average firing rates in PBC-region neurons abruptly increased to 89 ± 21% of baseline at P14 ($p<0.05$ vs. P15; Figs. 2B, 3C) compared to 23-41% of baseline on the other postnatal days ($p=0.005$ for age effect). At postnatal day P12, there was a significant increase in PBC-region average firing rate from 23 ± 4% of baseline at P11 to 41 ± 7% at P12 ($p<0.05$; Fig. 3C). The average firing rate increased from 41 ± 7% at P13 to 89 ± 21% at P14, but this was not significant ($p=0.21$). The average firing rate abruptly decreased the next day to 35 ± 8% at P15 ($p=0.014$). Categorizing PBC-region neuron average firing rates showed that the percent of pentobarbital-resistant neurons abruptly increased from 14% at P13 to 33% at P14, followed by a decrease to 19% at P15 (Fig. 3D). In contrast, only 5% of neurons from P11 rats were pentobarbital-resistant and 81% were pentobarbital-sensitive; there were no neurons with intermediate pentobarbital sensitivity (Fig. 3D). Thus, PBC-region neurons had increased sensitivity to pentobarbital at P11 and increased resistance to pentobarbital at P14.

Pentobarbital resistance decreased in XII motoneurons during the critical developmental period

For XII motoneurons, pentobarbital resistance was variable with the 200 μM pentobarbital application (Figs. 4A, 4B), but there was a clear age-dependent decrease with a minimum at day P14 with the 300 μM pentobarbital application (Figs. 4C, 4D). For example, with the 200 μM pentobarbital application, XII motoneuron average firing rates increased significantly from day P12 (41 ± 12%) to day P13 (77 ± 13%; $p<0.05$), and then decreased significantly from day P13 to P14 (61 ± 8%; $p<0.05$; Fig. 4A). There was a second significant increase in XII motoneuron firing rates from day P15 (56 ± 8%) to day P20 (84 ± 10%; $p<0.001$ at P20; $p<0.001$ for age effect; Fig. 4A). In general, there was a decrease in the percentage of pentobarbital-resistant XII motoneurons during the critical period with 37% of neurons

resistant at P10, 12% resistant at P12, and then 51% resistant 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.

During the 300 μM pentobarbital application, XII motoneuron average firing rates progressively decreased from 61 ± 11% of baseline at P10 to 15 ± 3% of baseline at P14 before increasing up to 53 ± 6% of baseline at P20 ($p<0.001$ for age effect; Fig. 4C). Average firing rates in P11-P15 XII motoneurons were significantly lower than P20 rats ($p<0.05$; Fig. 4C). The percentage of pentobarbital-resistant XII motoneurons was 21-22% at P10 and P20 (*i.e.*, ages bracketing the critical period), but there was a trough with minimum of only 3% of resistant neurons 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 neurons (Fig. 4D). These data show that pentobarbital sensitivity increased dramatically in XII motoneurons during the critical developmental period.

Pentobarbital resistance in NTS neurons decreased at P14

In NTS, pentobarbital resistance was consistent during the 200 μM pentobarbital application (Figs. 5A, 5B), but decreased significantly at P14 during the 300 μM pentobarbital application (Figs. 5C, 5D). For example, NTS neuron firing rates decreased to 52-74% of baseline during the 200 μM pentobarbital application ($p=0.349$ for age effect; Fig. 5A). The only significant change was from P15 (52 ± 11%) to P20 (65 ± 7%; $p=0.015$; Fig. 5A). The percentage of pentobarbital-resistant NTS neurons was variable and did not show any obvious trends (Fig. 5B). The percentage of resistant NTS neurons was lowest with 11% at P11 and maximal with 35% at P20 (Fig. 5B). The percentages of intermediate and sensitive NTS neurons were relatively unaltered from P10-P20.

During the 300 μM pentobarbital application, NTS neuron average firing rates ranged between 23-54% of baseline with the highest rate of 54 ± 7% of baseline at P13 ($p<0.001$ for age effect; Fig. 5C). The average firing rate significantly decreased to 23 ± 8% of baseline at P14 and then significantly increased to 37 ± 8% of baseline at P15 ($p<0.05$ for both changes; 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). NTS neurons decreased in pentobarbital resistance at P14 similar to the minimal pentobarbital resistance of XII motoneurons at P14.

Pentobarbital resistance in cortical neurons abruptly decreases at P12

Cortical neurons were generally more sensitive to pentobarbital than medullary neurons with an abrupt decrease in pentobarbital resistance at P12. During the 200 μM pentobarbital application, cortical neuron average firing rates ranged from 32-68% of baseline, except at P12 when the average firing rate decreased to 18% of baseline before returning to 38% of baseline at P13 ($p<0.004$ compared to P13; $p=0.019$ for age effect; Fig. 6A). There were no resistant cortical neurons at P12 and 89% of cortical neurons were pentobarbital-sensitive. P20 rats had the greatest percentage of resistant neurons at 30% and only 55% of cortical neurons were sensitive to pentobarbital (Fig. 6B). During the 300 μM pentobarbital application, cortical neuron average firing rates were lowest at P12 (14 ± 2%) and P20 (15 ± 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%.

Pentobarbital resistance varies across medullary regions during critical development period

In addition to abrupt day-to-day changes within specific medullary regions, there were also regional shifts in pentobarbital sensitivity. Before and after the critical period, at P10 and P20, XII motoneurons had significantly higher average firing rates compared to PBC-region neurons and compared to PBC-region, NTS, and cortical neurons, respectively ($p \leq 0.010$ for region effect; Figs. 7A, 7D) Further, the percentage of pentobarbital-resistant neurons was greatest in XII motoneurons at 22% compared to only 0-13% of PBC-region, NTS and cortical neurons from P10 and P20 rats during 300 μM pentobarbital (Figs. 3D, 4D, 5D, 6D). Together, these data suggest that before and after the critical period, XII motoneurons were the most pentobarbital-resistant.

During the critical period, however, pentobarbital resistance shifted between brain regions. For example, in P13 rats, NTS neurons were the most resistant to pentobarbital. NTS neuron average firing rates were significantly higher than in XII or CTX neurons ($p<0.05$ compared to NTS; $p<0.001$ for region effect; Fig. 7B). Further, 21% of NTS neurons were pentobarbital-resistant, while only 2-3% of XII and cortical neurons and 14% of PBC-region neurons were pentobarbital-resistant (Figs. 3D-6D). The next day, at P14, PBC-region neurons were the most pentobarbital-resistant. The percentage of pentobarbital-resistant PBC-region neurons was 33% while only 3-5% of NTS, XII, and cortical neurons were pentobarbital-resistant (Fig. 3D-6D). Likewise, PBC-region neurons from P14 rats had higher average firing rates compared to NTS and XII neurons ($p<0.05$ for XII and NTS neurons compared to PBC-region neurons; $p<0.001$ for region effect; Fig. 7C).

V. DISCUSSION

In acutely isolated medullary and cortical slices *in vitro*, neuronal sensitivity to bath-applied pentobarbital changed significantly during the critical developmental period in an age- and region-dependent manner. Pentobarbital was used to pharmacologically test for epsilon subunit incorporation, similar to previous studies from our group (Hengen *et al.*, 2009, 2011, 2012). This study, however, is the first to demonstrate functional epsilon subunit expression in respiratory-related medullary regions each day during the critical period. Our results suggest that epsilon subunit expression dynamically adjusts during development in individual regions, instead of uniformly changing throughout the respiratory control system. Further, this is the first study to suggest that epsilon subunit expression does not increase in parallel with brain allopregnanolone concentrations, and may be regulated by other factors instead. Thus, epsilon subunit incorporation in $GABA_A$Rs may have additional roles in native receptors beyond conferring resistance to positive allosteric modulation.

Increased neuronal inhibition during the critical period in respiratory control development

The critical period in respiratory control development represents a shift 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 the critical period, the balance of neuronal excitation *versus* inhibition appears to shift towards increased inhibition. For example, glutamate concentrations and NMDA receptor subunits NR1 and NR2A expression decrease significantly while GABA, $GABA_B$ receptor, and glycine receptor expression increases in PBC, XII, and NTS neurons (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) at P12 in PBC, XII and NTS neurons (Liu and Wong-Riley, 2012). Electrophysiological data also suggest increased inhibition in XII motoneurons at P12-13 because: (1) amplitude and frequency of spontaneous EPSCs is significantly decreased while the amplitude and frequency of spontaneous IPSCs is increased; (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 $GABA_A$Rs, the subunit composition switches from predominately expressing alpha-3 subunits to predominately expressing alpha-1 subunits in PBC 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 during the critical period. However, excessively inhibiting respiratory-related neurons could decrease respiratory drive and diminish airway patency. Compensatory mechanisms within the respiratory control system to offset increased neuronal inhibition are unknown.

Epsilon subunit expression dynamically adjusts during the critical period in a nuclei-specific manner

Epsilon subunit expression: Relatively little is known regarding the physiological role of epsilon subunits although $GABA_A$Rs containing epsilon subunits are found in cholinergic, dopaminergic, serotonergic, and noradrenergic neurons that have neuromodulatory actions (Moragues *et al.*, 2000; Belujon *et al.*, 2009). In adult rats, epsilon subunit mRNA is expressed throughout the rat brainstem including the raphe nuclei, A5 area, locus coeruleus, dorsal vagal complex, and cortex (Moragues *et al.*, 2000; Hengen *et al.*, 2012, Turner *et al.*, in preparation) Interestingly, epsilon subunit mRNA has increased expression in respiratory-related areas such as the PBC-region, XII motor nucleus, NTS, and ventral respiratory column compared to non-respiratory medullary regions (lateral trigeminal) or the

cortex (Turner *et al.*, in preparation). Epsilon subunit expression may also have a developmental role as epsilon mRNA expression steadily increases from embryonic day 14 (E14) to postnatal day 12 (P12) in rat medullas (Pape *et al.*, 2009) and functional epsilon subunits are found in the NTS neurons from P13-P45 rats (Kasparov *et al.*, 2001). Thus, epsilon subunits are localized to specific brain regions during embryogenesis through to adulthood, but their precise function is poorly understood with respect to development, neuromodulation, or respiratory control.

Role of epsilon subunits in native receptors: From one perspective, epsilon subunits in $GABA_AR$s are hypothesized to confer resistance to endogenous increases in brain allopregnanolone concentrations in respiratory-related neurons (Hengen *et al.*, 2012; Turner *et al.*, in preparation). Currently, there are two main models of naturally increased epsilon subunit expression: hibernation and pregnancy. During the torpor phase of hibernation, cortical neurons are electrically silent, yet the brainstem continues to regulate cardiorespiratory function (Drew *et al.*, 2001, 2007). The mechanisms underlying this maintenance of cardiorespiratory control are poorly understood. Epsilon subunits appear to contribute to cardiorespiratory function during the hibernation cycle of thirteen-lined ground squirrels because NTS neurons and neurons within the region of the ventral respiratory column have increased resistance to pentobarbital compared to summer active ground 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). These data suggest that epsilon subunits contribute to maintaining cardiorespiratory-related neuronal excitability.

Increased epsilon subunit expression also appears to help maintain respiratory-related function during late pregnancy (Hengen *et al.*, 2012; Turner *et al.*, in preparation) when central allopregnanolone

concentrations increase 3-4 fold (Concas *et al.*, 1998). Specifically, respiratory motor output on the phrenic nerve continues significantly longer following sequential pentobarbital injections in pregnant rats compared to non-pregnant female rats (Hengen *et al.*, 2012). In these experiments, respiratory frequency is preserved rather than phrenic burst amplitude, suggesting the PBC neurons increase epsilon subunit expression. Consistent with this hypothesis, neurons in the PBC-region of acutely isolated medullary slices from pregnant rats are more resistant to bath-applied pentobarbital and there is increased epsilon subunit immunoreactivity in PBC neurons from pregnant rats compared to non-pregnant female and male rats (Hengen *et al.*, 2012). Likewise, XII motoneurons from pregnant rats are also more resistant to pentobarbital and allopregnanolone *in vitro* compared to male rats (Turner *et al.*, in preparation). Similar to the hibernation studies, cortical neurons remain pentobarbital-sensitive and do not increase epsilon subunit immunoreactivity (Hengen *et al.*, 2012). Thus, these data suggest that epsilon subunit expression increases in respiratory-related brain regions to protect breathing from excessive inhibition. However, it remain unknown whether epsilon subunit expression increases during other physiologically stressful periods when allopregnanolone increases, such as during the critical period in respiratory control development during postnatal days P11-P14 in rats.

The present study demonstrated that epsilon subunit incorporation in $GABA_ARs$ dynamically changes during the critical period in an age- and region-dependent manner. Specifically, epsilon subunit expression abruptly increased at P14 in PBC-region neurons and decreased at P14 in NTS and XII neurons, as evidenced by changes in pentobarbital sensitivity. In PBC-region neurons, the percentage of resistant cells increased to 33% compared to only 3-5% in NTS, XII, and cortical neurons. Thus, the percentage of resistant cells in the PBC-region is 6-10-fold higher than in other medullary, respiratory-related regions and also non-respiratory cortical neurons at P14. With respect to NTS neurons, the percentage of pentobarbital-resistant neurons was 21% at P13 and dropped to only 5% at P14,

suggesting that the NTS has the greatest epsilon subunit expression at P13 (rather than at P14 as in PBC-region neurons). Paradoxically, XII motoneurons gradually decreased pentobarbital resistance from P11-P15, although by P20 pentobarbital resistance returned to P10 levels (~22% of XII neurons). Thus, these data suggest that medullary epsilon subunit incorporation in $GABA_A$Rs may play a role in regulating respiratory-related neuronal excitability during the critical period in a region specific manner. These findings agree with mRNA data from pregnant and male rats showing that epsilon subunit mRNA varies significantly between respiratory-related areas, although mRNA expression is generally higher in respiratory-related areas compared to non-respiratory medullary or cortical regions (Turner *et al.*, in preparation). The reasons for the abrupt increase or decrease in pentobarbital-resistance (and presumably epsilon subunit expression) are not clear. The hypothesis that all respiratory-related neurons, such as PBC-region neurons and XII motoneurons, will alter epsilon subunit expression together in parallel at the same time is not supported by our data. Instead, this study suggests that multiple factors regulate epsilon subunit incorporation during the critical developmental period.

Cortical neurons were generally highly sensitive to pentobarbital during the 300 μM 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 $GABA_A$R subunits. For example, the abrupt increase in pentobarbital sensitivity at P12 in cortical neurons may be due to increased expression of $GABA_A$R 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 $GABA_A$R modulation by flunitrazepam in P12 rats compared to P7 rats (Grobin and Morrow, 2001). 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) and are therefore insensitive to benzodiazepines. Thus, 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.

Additional unique characteristics of epsilon subunits have been described in recombinant epsilon-containing $GABA_AR$. For example, epsilon subunits are hypothesized to confer spontaneous opening (GABA-independent tonic current), insensitivity to benzodiazepines, slow receptor deactivation, altered receptor desensitization, and negative modulation by anabolic androgenic steroids (Davies *et al.*, 1997, 2001; Whiting *et al.*, 1997; Thompson *et al.*, 1998, 2002; Neelands *et al.*, 1999; Maksay *et al.*, 2003; Wagner *et al.*, 2005; Jones *et al.*, 2006) in addition to resistance to positive allosteric modulation (Irnaten *et al.*, 2002; Wagner *et al.*, 2005). However, some of these characteristics vary widely between epsilon subunit constructs (Thompson *et al.*, 2002; Jones *et al.*, 2007). Therefore, carefully testing the expression of the traits in native epsilon-containing $GABA_ARs$ is an important direction for future studies. Determining the unique characteristics of naturally occurring epsilon-containing $GABA_AR$ will lead to a better understanding of epsilon subunit incorporation's physiological impacts.

Potential mechanisms for regulating epsilon subunit incorporation: We hypothesized that incorporation of epsilon subunits in medullary regions**,** especially respiratory-related neurons, would increase in parallel with increased brain allopregnanolone concentrations. However, when cortical allopregnanolone levels are 3-4 fold higher at P10 and P14 (compared to P8 and P15 levels; Grobin and Morrow, 2001), pentobarbital resistance (and presumably epsilon subunit expression) spiked abruptly for only one day in PBC-region neurons and NTS neurons, and there was a long-lasting increase pentobarbital sensitivity in XII motoneurons at P11-P15. Since allopregnanolone concentrations in large samples of medullary and cortical tissue change in parallel (Hirst *et al.*, 2006; Billiards *et al.*, 2006), this

hypothesis appears to be falsified and epsilon subunit expression is likely regulated by other unknown factors. Alternatively, allopregnanolone levels may vary and be regulated at the microscopic level within medullary regions, and epsilon expression may still be correlated with changes in local allopregnanolone levels.

Finally, increased allopregnanolone levels may be sufficient, but not necessary, to increase epsilon subunit expression. Another potential mechanism that could change epsilon subunit expression is the intracellular chloride ion concentration ($[Cl^-]_i$). Interestingly, when $[Cl^-]_i$ is high, $GABA_A$Rs express alpha-3 subunits and use the NKCC1 chloride ion transporters (Succol *et al.*, 2012). This phenotype is similar to the $GABA_A$R in PBC-region and NTS neurons in rats before the onset of the critical period (Liu and Wong-Riley, 2004, 2006, 2012). In contrast, when $[Cl^-]_i$ is low, then, alpha-3 subunit and NKCC1 chloride ion transporter expression decreases, while alpha-1 subunit and KCC2 chloride ion transporter expression increases (Succol *et al.*, 2012). Again, this change aligns with the phenotype described following the critical period in PBC-region and NTS neurons (Liu and Wong-Riley, 2004, 2006, 2012). Furthermore, when $[Cl^-]_i$ is low, $GABA_A$Rs also increase delta subunit expression and increase tonic inhibition (Succol *et al.*, 2012). While delta subunit expression each day during the critical period is unknown, delta subunits are expressed in XII motoneurons in P3-P15 rats and mediate tonic inhibition (Numata *et al.*, 2012). These studies suggest that $[Cl^-]_i$ determines expression of multiple $GABA_A$R subunits and may also modulate epsilon subunit expression. Additionally, changes in $[Cl^-]_i$ may drive the abrupt switches in subunit expression and chloride transporters during the critical period. We speculate that neuronal inhibition may differentiate during the critical period when neurons are morphing from immature and inefficient GABAergic inhibitory signaling that potentially lacks tonic inhibition to mature GABAergic signaling featuring efficient, fast, synaptic inhibition with a constant inhibitory tonic current.

Physiological and Clinical Significance

Altered ventilation during normoxia and hypoxia during the critical period: Baseline respiratory frequency in normoxic conditions gradually increases from P0 and peaks at P13; followed by a gradual decrease (Liu *et al.*, 2006). Moreover, the ratios of minute ventilation to oxygen consumption and to CO_2 production abruptly increase in normoxic conditions at P13 (Liu *et al.*, 2009). During acute hypoxia, the ventilatory response is the weakest in P13 animals, but is significantly lower from P12-P14 compared to the rest of the first three postnatal weeks (Liu *et al.*, 2006). Further, at P13 there is an inadequate metabolic rate and the ratios of minute ventilation to oxygen consumption and to CO_2 production are also compromised during acute hypoxia (Liu *et al.*, 2009). In contrast, these ratios remain stable during normoxia and hypoxia during the remaining second and third postnatal weeks (Liu *et al.*, 2009). Likewise, P12-13 male rats have lower ventilation during hypercapnia (Holley *et al.*, 2012). These changes suggest that the massive rearrangement of receptor and subunit expression and neurotransmitter concentrations have physiological consequences to the ability to respond to a stressor such as hypoxia, thereby highlighting the vulnerability of the respiratory control system during this time.

Translating neurodevelopmental age across species: Translating neurodevelopmental milestones across species for relative developmental ages is challenging and there are conflicting reports in the literature. One report suggests that for brain development, rat postnatal days P11-14 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 (early third trimester; Clancy *et al.*, 2007). In either case, understanding the endogenous mechanisms that create abrupt developmental changes and most importantly, regulate respiratory neuron excitability could have clinical implications for maintaining stable breathing.

Clinical implications for abrupt changes in respiratory control in human infants: If the P11-P14 rat developmental milestones translate most closely to a 2-4 month old human infant then this time frame correlates with the peak incidence of sudden infant death syndrome (SIDS) (Goldberg *et al.*, 1986). 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. If the P11-P14 rat developmental milestones translate most closely to an early third trimester fetus/pre-term infant then the critical period could have 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 in the respiratory control system. Thus, understanding the endogenous mechanisms that create abrupt developmental changes and stabilize the respiratory control system could have powerful clinical implications for maintaining stable breathing.

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Table 1: Numbers of neurons recorded for time control experiments. A total of 26 P10-P15 rats and 7 P20 rates were used for time control experiments to record spontaneous action potentials from 270 neurons. The distribution of neuronal recordings is shown.

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 from in pentobarbital-application experiments. A total of 61 young rats were used for pentobarbital application experiments to record spontaneous action potentials from 1,118 neurons. The distribution of neuronal recordings is shown.

Pentobarbital experiments	**P10** (n=6 rats)	**P11** (n=7 rats)	**P12** (n=8 rats)	**P13** (n=10 rats)	**P14** (n=11 rats)	**P15** (n=10 rats)	**P20** (n=9 rats)
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 is shown.

Pentobarbital- & muscimol-resistant neurons	**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

Figure 1: Recording sites. (A) Silicon multichannel electrodes were positioned in the shaded areas to extracellularly record spontaneous action potentials from PBC-region (top), NTS and XII neurons (center and bottom).

Fig. 1

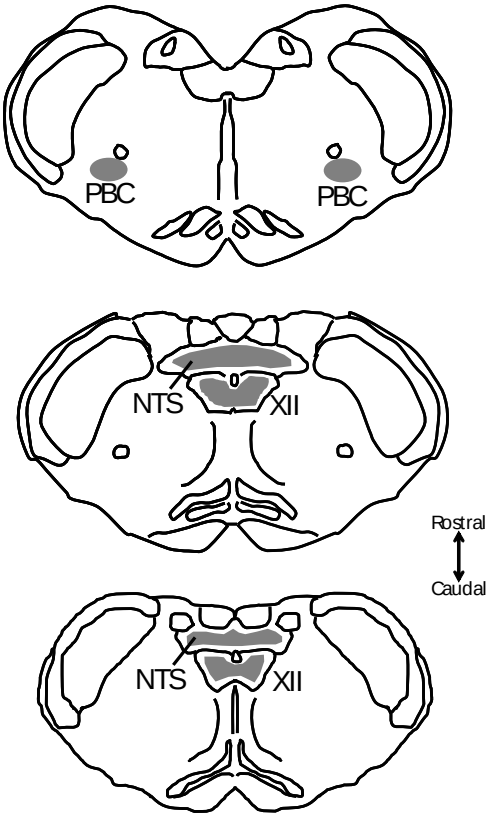


Figure 2: Spontaneous action potential firing rates were stable during time control experiments and decrease with pentobarbital application. (A) There were no time-dependent changes in neuronal activity during time control experiments during the critical period (black lines) in PBC-region (black squares), XII (white squares), NTS (silver squares) and cortical neurons (charcoal squares). Similarly, spontaneous neuronal activity was stable in PBC-region (gray diamonds), XII (gray triangles), NTS (gray circles) and cortical neurons (gray squares) from P20 rats (gray lines). Muscimol application at the end of the experiment inhibited neuronal firing rates. (B) Sample data are shown for pentobarbital (200 and 300 μM) application in PBC-region neurons from P10 (gray circles, gray line), P11 (white squares), P12 (silver squares), P13 (charcoal squares), P14 (black squares), P15 (gray triangles, gray line), and P20 (gray diamonds, gray line) rats. In general, pentobarbital application decreased neuronal firing rates ($p<0.05$ for drug effect). † indicates drug effect $p<0.05$.

Fig. 2

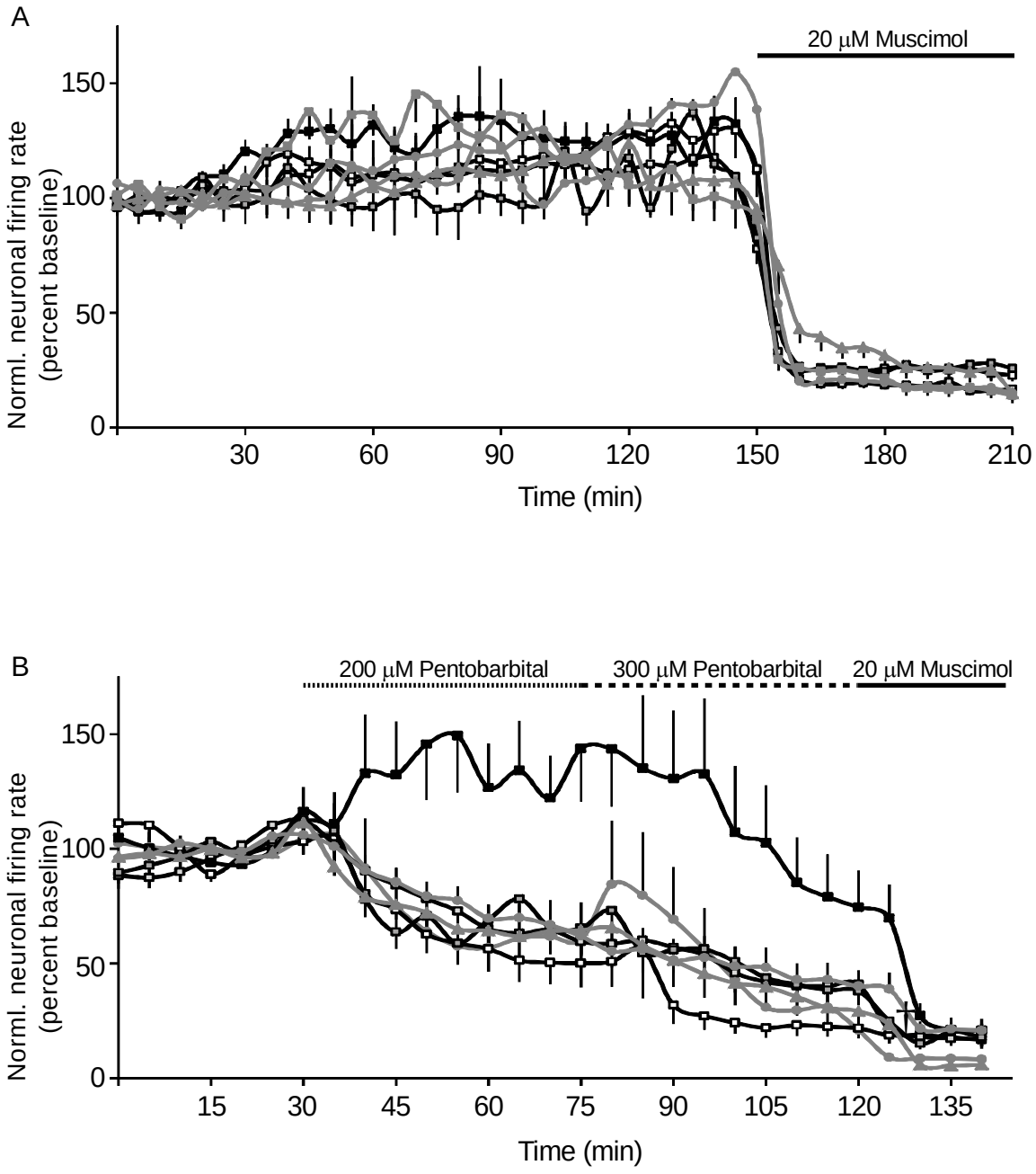

A
20 μM Muscimol
Norml. neuronal firing rate
(percent baseline)
150
100
50
0
30
60
90
120
150
180
210
Time (min)
B
200 μM Pentobarbital
300 μM Pentobarbital
20 μM Muscimol
Norml. neuronal firing rate
(percent baseline)
150
100
50
0
15
30
45
60
75
90
105
120
135
Time (min)

Figure 3: Pentobarbital resistance abruptly increases at P14 in PBC-region neurons. (A) In PBC-region neurons average firing rates from P10-P13 rats is 61-73% of baseline 200 μM pentobarbital application. At P14, the average firing rate abruptly increases to 133±22% of baseline before returning to 63% and 69% of baseline in PBC-region neurons from P15 and P20 rats ($p<0.05$ at P14; $p=0.002$ for age effect). (B) Classifying PBC-region neuron average firing rates from 200 μM pentobarbital application as sensitive (<40% of baseline; white), intermediate (40-80% of baseline; gray) or resistant (>80% of baseline; black) shows that the percentage of resistant cells steadily increases from 16% at P11 to 47% at P14. (C) With 300 μM pentobarbital application, PBC-region neuron average firing rates abruptly increase to 89±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) Classifying PBC-region neuron average firing rates into sensitive (white bars), intermediate (gray bars), and resistant (black bars) groups from the last 15 min of 300 μM pentobarbital application shows the PBC-region neurons resistant to pentobarbital abruptly increases from 14% at P13 to 33% at P14. Symbols are the same as in Fig. 3.

Fig. 3

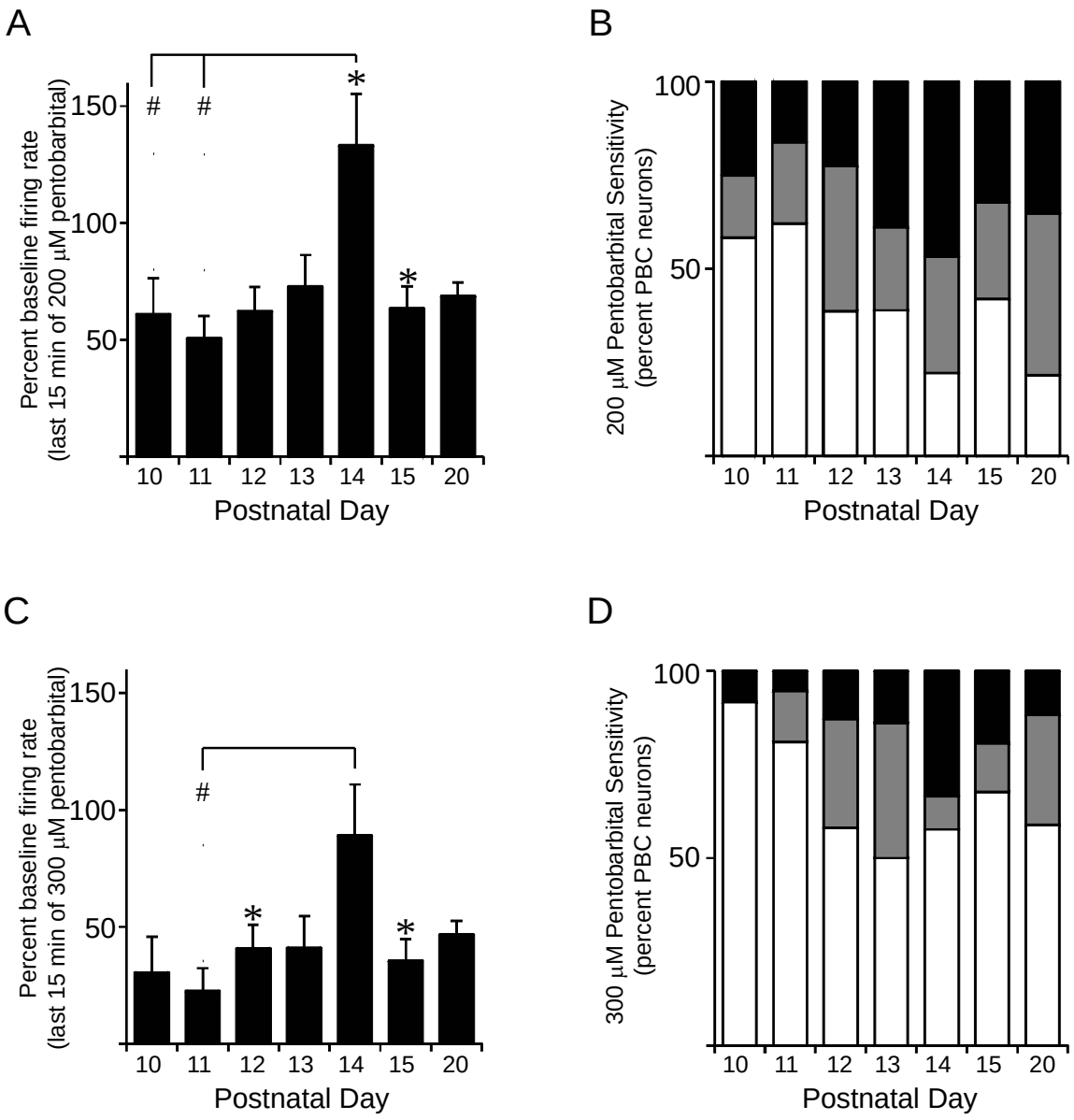

A
Percent baseline firing rate
(last 15 min of 200 μM pentobarbital)
150
100
50
#
#
*
*
10
11
12
13
14
15
20
Postnatal Day
B
200 μM Pentobarbital Sensitivity
(percent PBC neurons)
100
50
10
11
12
13
14
15
20
Postnatal Day
C
Percent baseline firing rate
(last 15 min of 300 μM pentobarbital)
150
100
50
#
*
*
10
11
12
13
14
15
20
Postnatal Day
D
300 μM Pentobarbital Sensitivity
(percent PBC neurons)
100
50
10
11
12
13
14
15
20
Postnatal Day

Figure 4: Pentobarbital sensitivity increased in XII motoneurons during the critical period. (A) Average firing rates in XII motoneurons decreased from 72 ± 10% of baseline at P10 to only 41-49% of baseline at P11-12 before increasing to 77 ± 13% of baseline at P13 ($p<0.05$ at P13; $p<0.001$ for age effect) during the last 15 min of 200 μM pentobarbital. (B) Classifying XII motoneuron average firing as sensitive (<40% of baseline; white), intermediate (40-80% of baseline; gray) or resistant (>80% of baseline; black) shows that at P10 37% of cells were resistant to pentobarbital, however, resistance decreased to only 12% of cells at P12. (C) During 300 μM pentobarbital application XII motoneurons average firing rates decreased from 61 ± 11% of baseline at P10 to only 15 ± 3% of baseline at P14 before returning to 53 ± 6% of baseline at P20 ($p<0.001$ for age effect). (D) Classifying XII motoneuron average firing rates during into 300 μM pentobarbital application as sensitive (white bars), intermediate (gray bars), and resistant (black bars) shows that in P10 and P20 rats 22% of cells were resistant to pentobarbital, however, resistance decreased to only 3-9% of cells during the critical developmental period from P11-P15. Symbols are the same as in Fig. 3.

Fig. 4

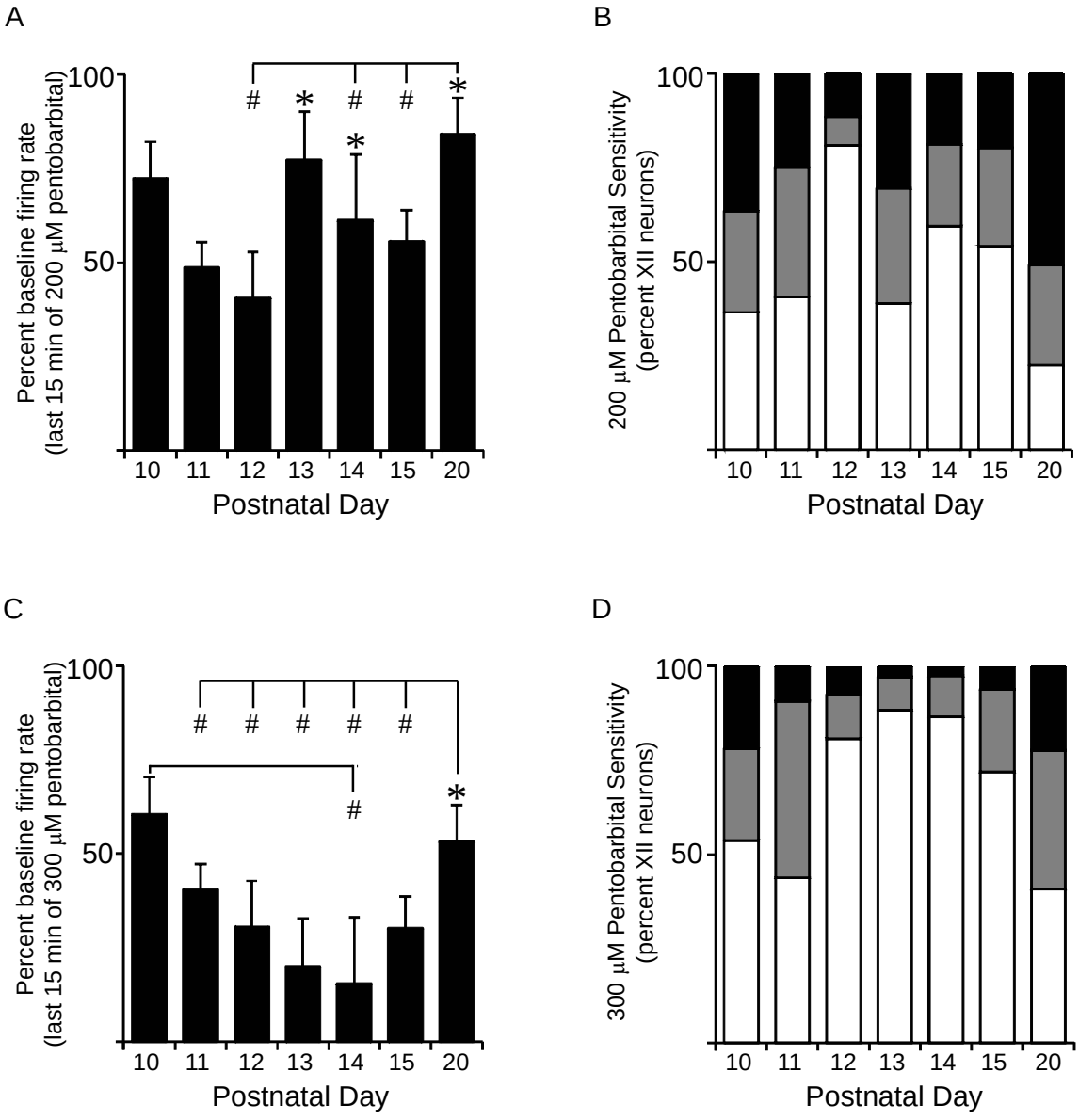

A
Percent baseline firing rate
(last 15 min of 200 μM pentobarbital)
100
50
10 11 12 13 14 15 20
Postnatal Day
B
200 μM Pentobarbital Sensitivity
(percent XII neurons)
100
50
10 11 12 13 14 15 20
Postnatal Day
C
Percent baseline firing rate
(last 15 min of 300 μM pentobarbital)
100
50
10 11 12 13 14 15 20
Postnatal Day
D
300 μM Pentobarbital Sensitivity
(percent XII neurons)
100
50
10 11 12 13 14 15 20
Postnatal Day

Figure 5: Pentobarbital abruptly decreased at P14 in NTS neurons. (A) Pentobarbital application (200 μM) decreased NTS neuron average firing rates to 52-74% ($p=0.349$ for development effect). P15 rats had lower average firing rates at 52 ± 11% of baseline compared to P20 rats with average firing rates of 65 ± 7% ($p<0.05$). (B) Classifying NTS neurons as sensitive (<40% of baseline; white), intermediate (40-80% of baseline; gray) or resistant (>80% of baseline; black) shows that in P11 rats only 11% of cells were resistant whereas pentobarbital resistance was found in 26% and 35% of cells in P13 and P20 rats, respectively. (C) Similarly, 300 μM pentobarbital application decreased NTS neuron average firing rates to 23-54% of baseline ($p<0.001$ for age effect). Average firing rates were the highest in P13 rats at 54 ± 7% of baseline and the lowest in P14 rats at 23 ± 8% of baseline ($p<0.05$). (D) Classifying NTS neurons into sensitive (white bars), intermediate (gray bars), and resistant (black bars) groups from the last 15 min of 300 μM pentobarbital application shows that at P13, 21% of NTS neurons were resistant, however, resistance abruptly decreased to only 5% of cells at P14. *indicates $p<0.05$ for t-test results; † indicates $p<0.05$ for age effect with Kruskal-Wallis ANOVA on ranks; # indicates $p<0.05$ for Dunn's post-hoc analysis.

Fig. 5

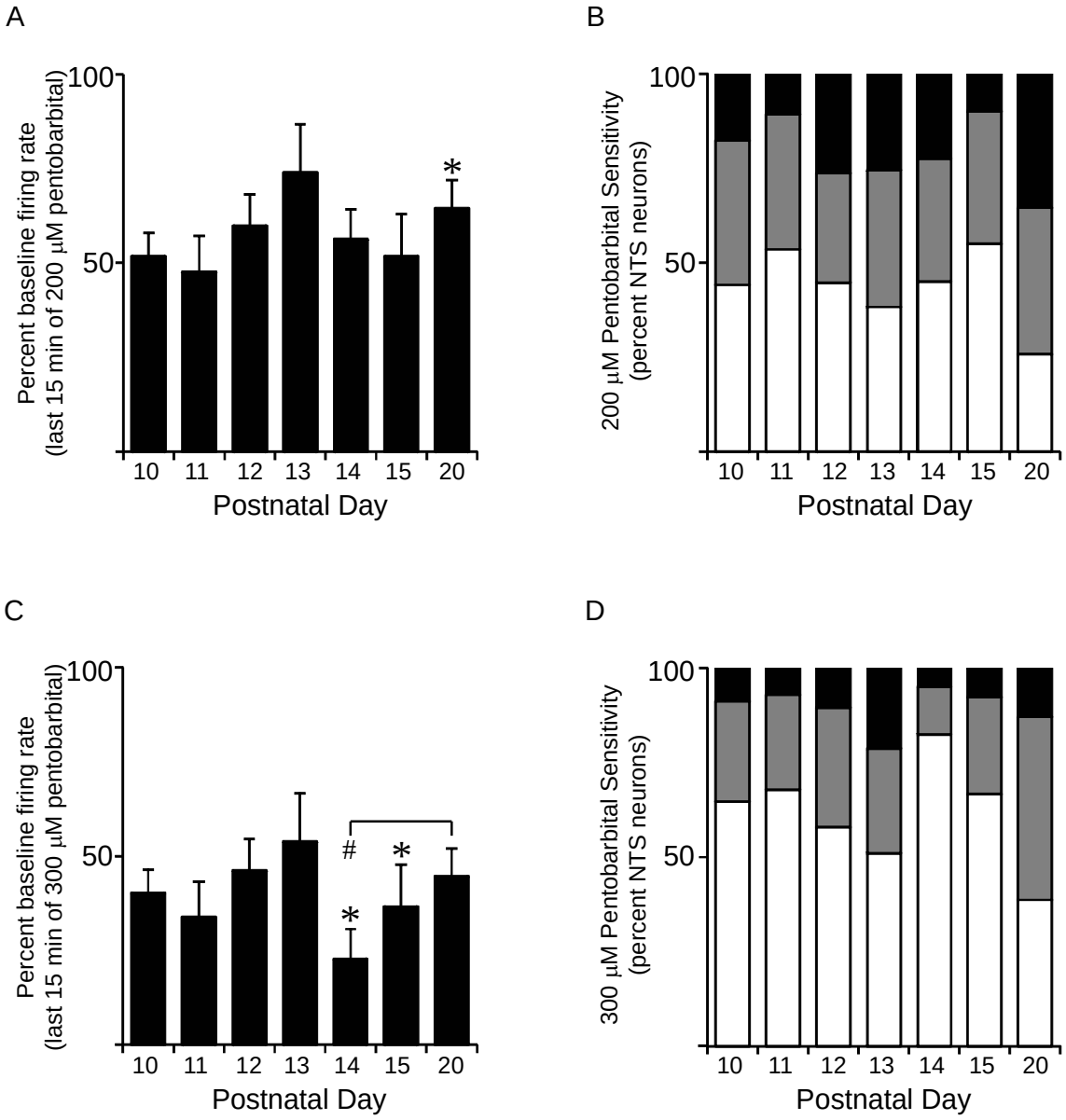

A
Percent baseline firing rate
(last 15 min of 200 μM pentobarbital)
100
50
10 11 12 13 14 15 20
Postnatal Day
*
B
200 μM Pentobarbital Sensitivity
(percent NTS neurons)
100
50
10 11 12 13 14 15 20
Postnatal Day
C
Percent baseline firing rate
(last 15 min of 300 μM pentobarbital)
100
50
10 11 12 13 14 15 20
Postnatal Day
#
*
*
D
300 μM Pentobarbital Sensitivity
(percent NTS neurons)
100
50
10 11 12 13 14 15 20
Postnatal Day

Figure 6: Pentobarbital sensitivity abruptly increased at P12 in cortical neurons. (A) In cortical neurons, 200 μM pentobarbital application decreased average firing rates to 32-68% of baseline, except at P12 when the average firing rate abruptly decreases 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 200 μM pentobarbital application into sensitive (<40% of baseline; white), intermediate (40-80% of baseline; gray) or resistant (>80% of baseline; black) shows that at P12 there were no resistant neurons whereas P20 had the greatest percentage of resistant neurons (30%). (C) During 300 μM 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) Cortical neuron classification into sensitive (white bars), intermediate (gray bars), and resistant (black bars) groups revealed that there were no resistant neurons at P12 or P20. Symbols are the same as in Fig. 3.

Fig. 6

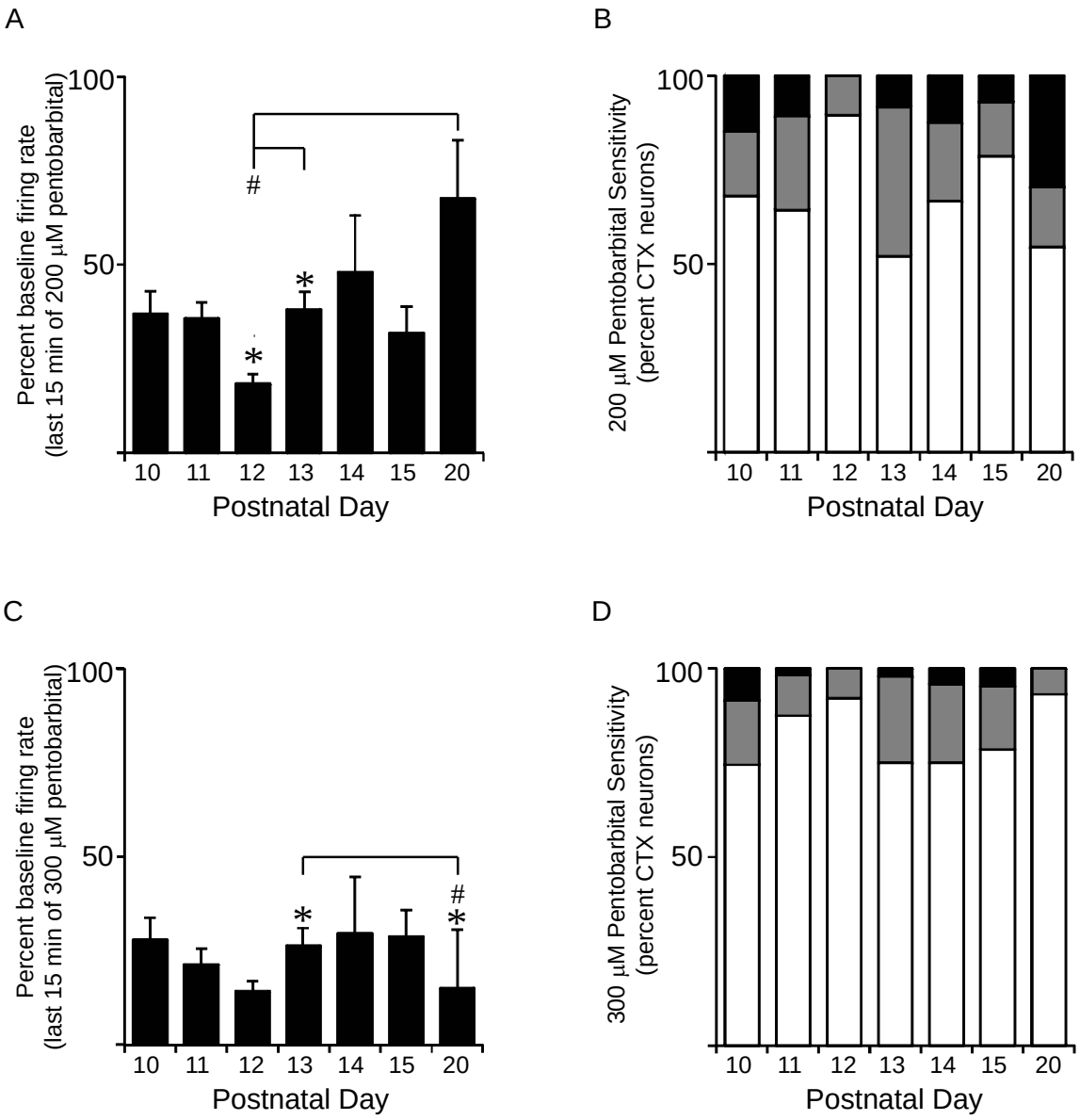
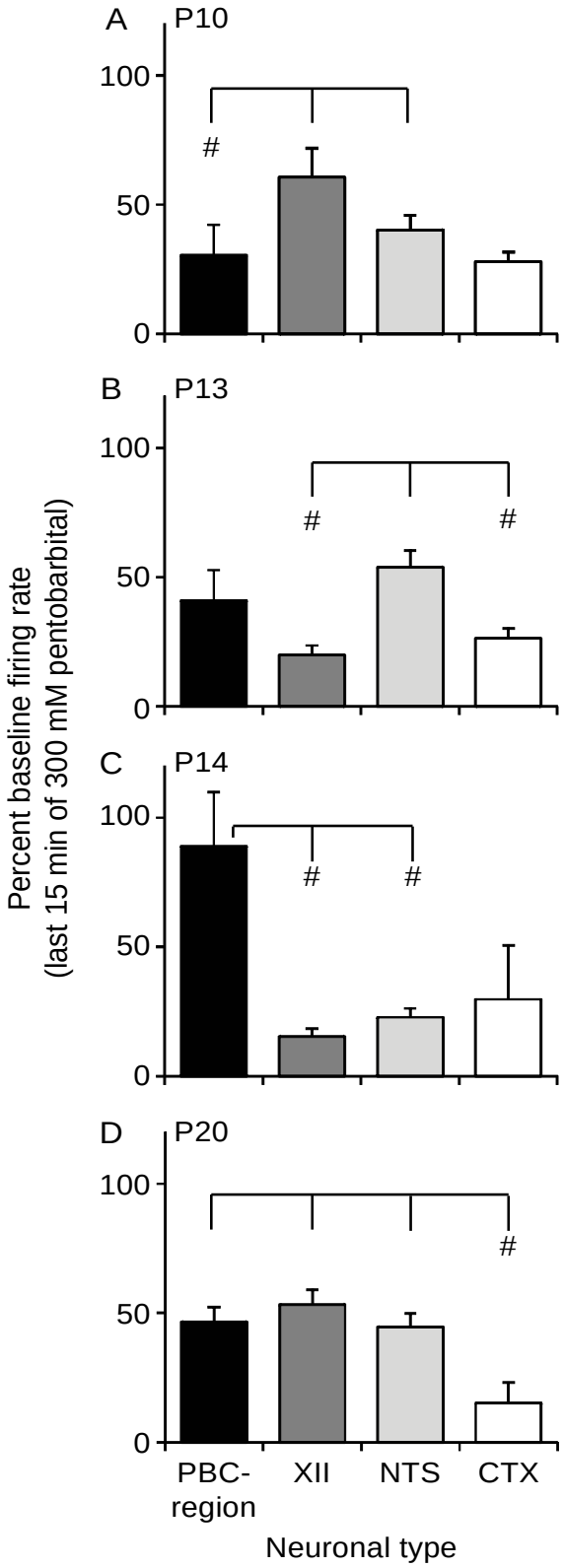

A
B
C
D
Percent baseline firing rate
(last 15 min of 200 µM pentobarbital)
200 µM Pentobarbital Sensitivity
(percent CTX neurons)
Percent baseline firing rate
(last 15 min of 300 µM pentobarbital)
300 µM Pentobarbital Sensitivity
(percent CTX neurons)
100
50
10 11 12 13 14 15 20
Postnatal Day
#
*

Figure 7: The most pentobarbital resistant brain region shifted during the critical period. (A) In P10 rats, XII motoneurons (charcoal bars) were more resistant to 300 μM pentobarbital application than NTS (black bars), PBC-region (silver bars) and cortical neurons (white bars; $p \leq 0.010$ for region effect). (B) In P13 rats, NTS neurons had the highest average firing rate during 300 μM pentobarbital application at 54 ± 7% of baseline, while PBC-region neurons decreased to 41 ± 7%, while both XII motoneurons and cortical neurons were significantly lower at only 20 ± 4% and 26 ±3 % of baseline, respectively ($p<0.001$ for region effect). (C) In P14 rats, PBC-region neuron (silver bars) average firing rates were 89 ± 21% of baseline during 300 μM pentobarbital application. In contrast, in NTS (black bars), XII (charcoal bars) and cortical (white bars) neuron normalized average firing rates were only 15-30% of baseline ($p<0.001$ for region effect). (D) In P20 rats, 300 μM pentobarbital application decreased average firing rates in NTS, XII, and PBC-region neurons to 45-53% of baseline, while firing rates in CTX neurons decreased to only 15 ± 3% of baseline ($p<0.05$ compared to medullary regions; $p<0.001$ for region effect). Symbols are as in Fig. 3.

Fig. 7


A
P10
100
50
0
#
B
P13
100
50
0
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C
P14
100
50
0
#
#
D
P20
100
50
0
#
PBC-region
XII
NTS
CTX
Neuronal type
Percent baseline firing rate
(last 15 min of 300 mM pentobarbital)

Chapter 4:

Delta-opioid receptor (DOR) activation prolongs respiratory motor output during oxygen-glucose deprivation (OGD) in neonatal rat spinal cord *in vitro*

Sara M. F. Turner and Stephen M. Johnson

I. ABSTRACT

Delta opioid receptor (DOR) activation protects the adult mammalian brain during oxygen-glucose deprivation (OGD), but it is not known whether neonatal spinal motor circuits are also protected. Also, it is unclear whether the timing of spinal DOR activation relative to spinal OGD is important for neuroprotection. Thus, a split-bath *in vitro* neonatal rat brainstem/spinal cord preparation was used to record spontaneous respiratory motor output from cervical (C4-C5) and thoracic (T5-T6) ventral spinal roots while exposing only the spinal cord to OGD solution (0 mM glucose, bubbled with 95% N_2 / 5% CO_2) or DOR agonist drugs (DADLE, DPDPE). Spinal OGD solution application caused respiratory motor output frequency and amplitude to decrease until all activity was abolished (*i.e.*, end-point times) after 25.9 ± 1.4 min (cervical) and 25.2 ± 1.4 min (thoracic). Spinal DOR activation via DPDPE (1.0 μM) prior-to and during spinal OGD increased cervical and thoracic end-point times to 35-48 min. Spinal DADLE or DPDPE (1.0 μM) application 15 min following spinal OGD onset increased cervical and thoracic end-point times to 36-45 min. Brief spinal DPDPE (1.0 μM) application for 10 min at 25 min before spinal OGD onset increased cervical and thoracic end-point times to 41-46 min. Overall, the selective DOR agonist, DPDPE, was more effective at increasing end-point times than DADLE. Naltrindole (DOR antagonist; 10 μM) pretreatment blocked DPDPE-dependent increase in end-point times, suggesting that DOR activation was required. Spinal naloxone (1.0 μM) application before and during spinal OGD also increased end-point times to 31-33 min, but end-point times were not altered by MOR activation or DOR activation/MOR blockade, indicating that there are complex interactions between OGD and opioid signaling pathways. These data suggest DOR activation before, during, and after spinal OGD protects central motor networks and may provide neuroprotection during unpredictable perinatal ischemic events.

II. INTRODUCTION

Oxygen-glucose deprivation (OGD) initiates a deleterious cascade of neuronal depolarization, glutamate release, Na^+ and Ca^{2+} ion influx, acute excitotoxicity, and long-lasting neuronal damage (Pugliese *et al.*, 2003; Bickler, 2004). Perinatal OGD affects 1 of 2300-5000 live births due to stroke, acute circulatory collapse, placental insufficiency, or cardiorespiratory failure (Badr and Purdy, 2006; Nelson, 2007; Rennie *et al.*, 2007). Perinatal OGD can also cause motor system disorders such as seizures, cerebral palsy, hypotonia, and apneas (Volpe, 2001; Badr and Purdy, 2006; Sotero de Menezes and Shaw, 2006; Nelson, 2007). Over 50% of full-term children with perinatal hypoxic-ischemic encephalopathy have mild to significantly delayed motor ability (van Schie *et al.*, 2007). Since no successful treatments exist (Badr and Purdy, 2006), novel strategies must be developed to protect CNS motor networks before, during and after perinatal OGD.

One strategy for providing neuroprotection against perinatal OGD is to apply principles learned from naturally hypoxia- and ischemia-resistant species (Borlongan *et al.*, 2004). Delta opioid receptors (DOR) are endogenous, evolutionarily conserved (Xia and Haddad, 2001), and activated during physiological stress (Hwang *et al.*, 1986). DOR activation is a potential mechanism for hypoxia/ischemia resistance because hypoxia-resistant red-eared slider turtles have more central DOR receptors compared to rats (Xia and Haddad, 2001). Also, endogenous DOR activation may contribute to neuroprotection in hibernating mammals during torpor when blood flow to the brain is severely reduced (Drew *et al.*, 2001, 2007). Thus, DOR activation in ischemia-sensitive mammals may be a powerful mechanism for inducing neuroprotection.

Accordingly, in cultured cortical or hippocampal neurons, DOR activation prior to excitotoxic glutamate application attenuates neuronal damage and death (Zhang *et al.*, 2006; Zhao *et al.*, 2006b). With respect to central motor networks, spinal OGD rapidly impairs motor function in swine *in vivo* (Lee

et al., 2008) and in rats *in vitro* (Jha *et al.*, 2003; Deshpande and Jha, 2004). However, intrathecal DOR agonist administration 9 min prior to spinal cord ischemia improves hind limb motor performance and reduces histological damage in spinal ventral horn (Horiuchi *et al.*, 2004). Although DOR activation provides neuroprotection in mature mammals, it is not known whether DOR activation protects neonatal spinal motor networks. Also, it's important to know whether DOR-dependent neuroprotection depends on the timing of DOR drug administration with respect to OGD onset, since perinatal ischemia is clinically unpredictable.

To address these questions, spontaneous respiratory motor output from cervical and thoracic ventral spinal rootlets was recorded in neonatal rat brainstem-spinal cord preparations *in vitro*. We tested whether respiratory spinal motor output was prolonged by the following conditions: 1) sustained spinal DOR activation 10 min prior-to and during spinal OGD, 2) spinal DOR activation at 10 min post-spinal OGD onset, and 3) brief spinal DOR activation for 10 min at 25 min before spinal OGD onset. This is the first study to illustrate the protective effects of DOR activation before, during, and after OGD in a neonatal spinal respiratory motor circuit. Preliminary results were published in abstract form (Freiberg *et al.*, 2008a,b).

III. METHODS

Brainstem-spinal cord preparations

The University of Wisconsin Institutional Animal Care and Use Committee approved all experimental procedures and all experiments conformed to International Guiding Principles for Biomedical Research

Involving Animals as established by the Council for International Organizations of Medical Sciences. Neonatal (P1-P3) Sprague-Dawley rats (n=138, Charles River, Wilmington, MA, USA) of either sex were anesthetized with 5% isoflurane (O_2 balance) before being decerebrated. The remaining tissue was placed in ice-cold artificial cerebrospinal fluid (aCSF), composed of (in mM): 120 NaCl, 26 $NaHCO_3$, 20 glucose, 2 $MgSO_4$, 1 $CaCl_2$, 3 KCl, and 1.25 Na_2HPO_4. The brainstem and spinal cord from the pontomedullary border to thoracic spinal segment T12 was removed in ice-cold aCSF and pinned down ventral side up in a standard recording chamber. A plastic and petroleum jelly barrier at C1 separated the chamber into a brainstem compartment (volume = 4.5 ml) and spinal compartment (volume = 8.5 ml) (Fig. 1A). The brainstem and spinal compartments were continuously bathed with oxygenated aCSF solution (26°C, aerated with 5% CO_2 and 95% O_2, pH = 7.4) at a flow rate of 7-8 ml/min in each compartment. During each experiment the brainstem compartment was always bathed with oxygenated aCSF. To expose the spinal cord to OGD, the spinal compartment was bathed with OGD solution, which was aCSF solution with 20 mM sucrose substituted for 20 mM glucose (aerated with 5% CO_2 and 95% N_2, pH = 7.4).

Electrophysiological recordings of spinal respiratory motor output

Spontaneously produced respiratory motor output was recorded by attaching glass suction electrodes to cervical (C_3-C_5; phrenic motoneurons) and thoracic (T_4-T_6; intercostal motoneurons) nerve roots. Signals were acquired at 50 Hz, amplified (10,000x) and band-pass filtered (0.1-500 Hz) using a differential AC amplifier (model 1700, A-M Systems, Everett, WA, USA) before being rectified and integrated (time constant = 50 ms) using a moving averager (MA-821/RSP, CWE, Inc., Ardmore, PA, USA; Fig. 1B). Data were collected using Axoscope hardware and software (Molecular Devices, Sunnyvale, CA, USA).

Brainstem-spinal cord preparations were allowed to equilibrate for 10-40 min before recording baseline data for 5 min. If respiratory spinal motor output decreased by >20% before spinal OGD application (without drug application), the experimental results were discarded. For all protocols with spinal OGD application, recordings were continued for >20 min after all spontaneous respiratory spinal motor activity was abolished. At the end of every experiment, the integrity of the barrier at spinal segment C1 was checked by injecting Chicago Blue dye into the brainstem compartment. If any dye flowed into the spinal compartment, the experiment results were discarded.

Oxygen measurements in the spinal compartment

Oxygen levels (PO_2) in the spinal compartment were measured with an oxygen electrode (ISO-OXY-2, World Precision Instruments, Sarasota, FL, USA) placed 1-2 mm lateral to the thoracic spinal cord at T5-T6 (contralateral to the suction electrode). Signals from the oxygen electrode were processed by an Apollo 4000 amplifier (World Precision Instruments) and recorded using Axoscope software. For calibration, the oxygen electrode was placed into the oxygenated aCSF and the OGD solution reservoirs for 5-10 min each prior to the experiment. The voltages measured in the reservoirs were set to be equal to the average reservoir PO_2 that was measured six different times with 1.0 ml samples withdrawn from the reservoir and injected into a blood-gas analyzer (ABL800 Flex, Radiometer America, Westlake, OH, USA). The PO_2 values were 593 ± 14 mm Hg and 57 ± 4 mm Hg in the oxygenated aCSF and OGD solution reservoirs, respectively. The bath was open to the air so that the PO_2 in the spinal compartment solution was larger than the value in the OGD solution reservoir (see Fig. 2F).

Experimental drugs

All drugs were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). DOR agonists included DPDPE ([D-Pen^{2,5}]-Enkephalin hydrate; DOR agonist), DADLE ([D-Ala^2, D-Leu^5]-enkephalin acetate salt; DOR agonist); DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; mu-opioid [MOR] agonist) antagonists included naloxone (opioid receptor antagonist), naltrindole (DOR antagonist) and 3β-naltrexol (MOR antagonist).

Data Analysis

Voltage traces of spinal respiratory motor output or "bursts" were analyzed using Clampfit software (Molecular Devices, Sunnyvale, CA, USA). Respiratory motor burst amplitude was measured at the peak of integrated nerve discharge and normalized to baseline. Thoracic burst frequency was measured as the number of thoracic respiratory bursts/min and graphed as percent change from baseline. Data were averaged into 5-min bins such that the 30-min time-point represents the averaged data from 25.0-29.9 min. Data were reported as mean ± SEM. One-way and two-way ANOVA's were performed with post-hoc comparisons using the Student-Newman-Keuls test in Sigma Stat software (Jandel Scientific Software, San Rafael, CA, USA). If normality or equal variance assumptions failed, data were transformed using the natural log or arcsine transforms (*e.g.*, ln transform was used for the cervical dose-response DADLE endpoints, thoracic dose-response DPDPE endpoints, and thoracic DADLE post-OGD onset endpoints; Arcsin square root was used for cervical and thoracic sustained DPDPE amplitude). $P<0.05$ was considered statistically significant.

IV. RESULTS

Spinal OGD abolishes spinal respiratory motor output

For time control experiments (n=8) in which the brainstem and spinal cord compartments were continuously bathed in aCSF solution, cervical burst amplitude decreased slowly over time to 75 ± 6% of baseline after 120 min ($p<0.05$ for 65-120 min; one-way RM ANOVA; Fig. 2B). In contrast, thoracic burst amplitude was unaltered over time ($p=0.95$; one-way RM ANOVA; Fig. 2C). Thoracic burst frequency was 94 ± 14% of baseline after 120 min ($p=0.93$; one-way RM ANOVA; Fig. 2D). Thus, brainstem-spinal cord preparations produced quantifiable, spontaneous respiratory motor output for 120 min with cervical burst amplitude decreasing gradually over time.

In separate experiments (n=11), baseline data were recorded (5 min), aCSF solution was spinally applied (10 min), and then OGD solution was spinally applied until all activity was abolished (Fig. 2A). Cervical and thoracic burst amplitudes were decreased compared to time controls at 20-30 min post-OGD onset ($p<0.001$; Figs. 2C, 2D). To test whether spinal burst area was correlated with burst amplitude, area was graphed versus amplitude using the 5-min bin data from each OGD experiment (this permits a wide range of data to be compared). Cervical and thoracic burst areas were tightly correlated with their respective amplitudes (cervical: slope = 1.00, $r^2=0.862$; thoracic: slope = 0.95, $r^2=0.838$). Cervical and thoracic motor activity was abolished at 25.9 ± 1.4 min (range=17.6-33.0 min; $p=0.027$ for drug effect) and 25.2 ± 1.4 min (range=17.3-32.6 min; $p<0.001$ for drug effect), respectively (Figs. 4A, 4B). Thoracic burst frequency (baseline = 4.2 ± 1.2 bursts/min) was decreased compared to time controls at 30-35 min post-OGD onset ($p=0.04$ for drug effect; Fig. 2E). Spinal compartment PO_2 was 458 ± 17 mm Hg during baseline, decreased to 165 ± 22 mm Hg after 10 min of spinal OGD, and was 98 ± 11 mm Hg after 30 min of spinal OGD (Fig. 2F).

To test whether OGD altered spinal burst shape, the time from burst onset to the time of burst peak was divided by burst duration to calculate percent time to the peak. In general, the percent time to the peak during baseline was less than 20% (*i.e.*, rapid onset and decrementing; Fig. 2B). For the OGD experiments, percent time to peak during baseline was 14 ± 6% (cervical) and 12 ± 4% (thoracic). During the last 20 spinal bursts, cervical percent time to peak increased to 29 ± 9% ($p=0.004$), while thoracic percent time to peak remained unchanged at 19 ± 5 ($p=0.11$). Thus, spinal OGD application did not dramatically alter spinal burst shape.

Spinal DOR activation prior-to and during spinal OGD prolongs respiratory motor output

DOR agonists (DADLE or DPDPE) were applied to the spinal cord for 10 min prior-to and during spinal OGD until spinal motor output was abolished (Fig. 3A). Since mean baseline thoracic burst frequencies were similar for OGD- and DOR agonist-treated preparations (3.4-4.5 bursts/min; $p>0.38$), frequency data were graphed as percent baseline frequency. DADLE (0.1 μM; n=3) application did not alter the OGD-induced frequency decrease (Fig. 3A). DADLE (1.0 μM; n=7) increased burst frequency compared to OGD at 30-55 min post-OGD onset ($p<0.002$) with 4/7 preparations active at 50 min post-OGD onset (Fig. 3A). DADLE (10 μM; n=5) increased burst frequency at 30-50 min post-OGD onset in 3/5 preparations ($p<0.04$; Fig. 3A). In 2/5 preparations, DADLE (10 μM) application abolished rhythmic activity before OGD onset (data excluded from Fig. 3A).

DPDPE (0.1 μM; n=5 or 10 μM; n=3) did not alter the OGD-dependent frequency decrease except for the 30-min time point for 0.1 μM DPDPE ($p<0.003$; Fig. 3B). DPDPE (1.0 μM; n=8) increased burst frequency compared to OGD data at 30-55, 65, and 75 min post-OGD onset with 3/8 preparations still active at 50 min post-OGD onset ($p=0.015$ for drug effect; Fig. 3B).

When respiratory motor output was quantified as end-point times (*i.e.*, time from OGD onset to time when spinal nerve motor activity was abolished) in OGD experiments, DADLE (0.1 μM) did not alter cervical (p=0.97) and thoracic (p=0.86) end-point times (Figs. 4A, 4B). DADLE (1.0 μM) did not alter cervical end-point times (34.0 ± 4.4 min; p=0.127; Fig. 4A), but increased thoracic end-point times to 48.5 ± 5.3 min (range=27.7-67.0 min; p<0.001; Fig. 4B). DADLE (10 μM; data from 3/5 preparations) prolonged cervical and thoracic end-point times to 55.1 ± 12.8 min (range=32.3-76.7 min; p=0.004; Fig. 4A) and 58.5 ± 9.0 min (range=49.3-76.5 min; p<0.001; Fig. 4B), respectively. DPDPE at 0.1 μM and 10 μM did not prolong cervical or thoracic end-point times (p>0.42; Figs. 4A, 4B). However, DPDPE (1.0 μM) prolonged cervical end-point times to 35.9 ± 3.9 min (range=22.7-52.0 min; p=0.035) and thoracic end-point times to 48.9 ± 9.7 min (range=22.6-103.9 min; p=0.007) (Figs. 4A, 4B). These data show that DADLE was ineffective at 0.1 μM, and DPDPE was ineffective at 0.1 and 10 μM. DADLE at 10 μM increased end-point times, but tended to abolish respiratory rhythm. Thus, amplitude data are shown only for 1.0 μM DADLE and DPDPE (Figs. 4C, 4D) and all subsequent experiments were performed at 1.0 μM.

With respect to cervical burst amplitude, DADLE (1.0 μM) had no effects, but DPDPE (1.0 μM) attenuated OGD-induced amplitude decrease at 15-30 min (p=0.016 for drug effect; Fig. 4C). For thoracic motor output, DADLE increased burst amplitude at 25-30 min (p<0.05; Fig. 4D), while DPDPE (1.0 μM) increased burst amplitude at 20-30 min post-OGD onset (p=0.019 for drug effect; Fig. 4D). Statistical comparisons were only performed during 5-30 min post-OGD onset because 15/17 preparations exposed to OGD alone were silent after 30 min.

Spinal DOR activation following spinal OGD onset prolongs respiratory motor output

DADLE (1.0 μM, n=7) or DPDPE (1.0 μM, n=7) was added to the spinal compartment 15 min following spinal OGD solution application. DADLE increased burst frequency (baseline = 4.0 ± 0.49 bursts/min) compared to OGD at 25-55 min post-OGD onset ($p=0.011$ for drug effect; Fig. 5A). DADLE increased cervical and thoracic end-point times to 40.7 ± 6.3 min (range=23.6-65.9 min; $p=0.008$) and 45.3 ± 7.1 min (range=26.1-73.1 min; $p<0.001$), respectively (Figs. 5C, 5D). DADLE did not alter the OGD-induced decrease in cervical and thoracic burst amplitudes (Figs. 5E, 5F). DPDPE increased thoracic burst frequency (baseline = 4.4 ± 0.62 bursts/min) at 25-50 min post-OGD onset ($p<0.001$ for drug effect; Fig. 5B) and increased cervical and thoracic end-point times to 36.2 ± 2.0 min (range=23.6–65.9 min; $p<0.001$) and 41.4 ± 3.6 min (range=26.1–73.1 min; $p<0.001$), respectively (Figs. 5C, 5D). DPDPE increased cervical burst amplitude at 25-30 min and thoracic burst amplitude at 20-25 min post-OGD onset (Figs. 5E, 5F).

Brief spinal DOR activation before OGD prolongs spinal respiratory motor output

To test whether DOR activation induces long-lasting effects, DADLE (1.0 μM; n=9) or DPDPE (1.0 μM; n=6) was applied to the spinal cord for 10 min, followed by aCSF solution for 25 min, and then OGD solution until 20 min after motor output was abolished (Figs. 6A, 6B). To control for potential time-dependent effects on respiratory motor output, separate OGD control experiments (n=6) were performed in which OGD solution was applied to the spinal cord after a 40-min baseline period. Similar to the OGD experiments in Fig. 2, OGD abolished cervical and thoracic bursts at 24.3 ± 1.7 min (range=20.3–31.9 min) and 26.8 ± 1.8 min (range=20.9-32.7 min), respectively (Figs. 6C, 6D).

DADLE did not alter OGD-induced changes in thoracic burst frequency (baseline = 4.5 ± 0.33 bursts/min; Fig. 6A) or thoracic end-point times (34.6 ± 4.9 min; p=0.23; Fig. 6D), but increased cervical end-point times to 32.0 ± 2.0 min (range=23.5-41.2 min; p=0.018; Fig. 6C). DADLE did not alter spinal burst amplitude (Figs. 6E, 6F). DPDPE increased thoracic burst frequency (baseline = 4.3 ± 0.59 bursts/min) at 25-45 and 60 min post-OGD onset (p=0.015 for drug effect; Fig. 6B) and increased cervical and thoracic end-point times to 41.0 ± 2.5 min (range=31.3–49.6 min) and 46.0 ± 3.5 min (range=39.4–61.8 min), respectively (p<0.001 for both; Figs. 6C, 6D). DPDPE did not alter spinal burst amplitude (p>0.64; Figs. 6E, 6F).

DPDPE-dependent neuroprotection requires spinal DOR activation

To test whether DPDPE effects were due to DOR activation, naltrindole (10 μM; selective DOR antagonist) was continuously applied to the spinal cord 10 min prior to initiating a spinal DPDPE (1.0 μM) and OGD experiment similar to that shown in Fig. 3B (n=5). Naltrindole abolished the DPDPE-dependent prolongation of spinal respiratory motor output with cervical and thoracic end-point times of 24.5 ± 4.6 and 26.2 ± 3.6 min, respectively (p>0.72; Figs. 7A, 7B). To test whether ongoing endogenous spinal DOR activation in neonatal rat brainstem-spinal cord preparations is neuroprotective, naltrindole (10 μM) was applied to the spinal cord prior-to and during spinal OGD (n=7). Under these conditions, there were no differences in end-point times compared to OGD for cervical (20.8 ± 3.8 min; p=0.17) or thoracic (22.6 ± 4.2 min; p=0.49) respiratory motor output (Figs. 7A, 7B).

Role of endogenous spinal opioid receptors during spinal OGD

To test whether general blockade of spinal opioid receptors alters the response to spinal OGD, naloxone (1.0 μM; broad spectrum opioid receptor antagonist; n=8) was applied 10 min prior-to and during spinal OGD (Fig. 8A). Naloxone increased thoracic burst frequency (baseline = 4.5 ± 0.8 bursts/min) at 30-35 min post-OGD onset ($p<0.001$; Fig. 8A). Naloxone also prolonged cervical and thoracic motor end-point times to 31.7 ± 1.3 min (range=25.6-36.9 min; $p=0.008$) and 33.9 ± 1.6 min (range=30.1-41.5 min; $p<0.001$), respectively (Fig. 8B). Naloxone application did not alter cervical or thoracic spinal burst amplitude (Figs. 8C, 8D).

Do spinal MOR receptors play a role during spinal OGD?

Since naloxone preferentially blocks MOR, we further investigated the role of spinal MOR by applying specific MOR agonist and antagonist drugs to the spinal cord prior-to and during OGD. In one set of experiments (n=8), DAMGO (0.01 μM; MOR agonist) was spinally applied 10 min prior-to and during spinal OGD (see below for justification of DAMGO concentration). In one preparation, respiratory activity was abolished before the OGD onset; these data were excluded. In 7/8 preparations, DAMGO did not prolong cervical (28.6 ± 5.8 min; $p=0.58$) or thoracic (32.2 ± 7.2 min; $p=0.29$) endpoint times, respectively, compared to OGD only. In separate experiments (n=7), we tested whether the combination of DOR activation and MOR blockade were additive. Accordingly, naltrexol (10 μM; selective MOR antagonist) was applied to the spinal cord 10 min before initiating a spinal DPDPE (1.0 μM) and OGD experiment similar to that shown in Fig. 3B. The combination of naltrexol and DPDPE increased cervical and thoracic end-point times to 33.9 ± 3.3 min ($p=0.021$) and 34.1 ± 2.6 ($p=0.005$), respectively, compared to spinal OGD only. However, these end-point times were not different compared to

DPDPE/OGD alone ($p>0.19$). Thus, the naloxone-induced increase in end-point times during spinal OGD did not appear to involve MOR.

Time-dependent effects of spinal DOR and MOR activation on respiratory motor output

The ideal drug will not alter respiratory motor output while protecting against spinal OGD. However, while testing the higher concentrations of DADLE or DAMGO on the spinal cord, there were unexpected changes in respiratory burst frequency, or large decreases in spinal burst amplitude. To characterize potential time-dependent changes, drugs were dissolved in control aCSF and applied to the spinal cord for 90 min (or at least 20 min after activity was abolished in some cases). For example, spinal DADLE (10 μM) application abolished spontaneous respiratory motor output in 2/5 preparations as stated above in section 3.2. After spinal application of DADLE (1.0 μM; n=4) for 90 min, thoracic burst frequency was 94 ± 3% of baseline (baseline = 4.4 ± 0.6 bursts/min; $p=0.46$), but cervical and thoracic burst amplitudes decreased to 39 ± 13% and 34% ± 10% of baseline, respectively ($p<0.03$ for drug effect; data not shown). In contrast, when DPDPE (1.0 μM; n=4) was spinally applied for 90 min, thoracic burst frequency was not altered at 80 ± 7% of baseline (baseline = 4.8 ± 0.3 bursts/min; $p=0.36$ for drug effect), and amplitude was not altered at 74 ± 6% (cervical; $p=0.49$ for drug effect) and 96 ± 12% of baseline (thoracic; $p=0.90$ for drug effect; data not shown).

With respect to MOR, spinal application of DAMGO (1.0 μM; n=3) abolished cervical and thoracic respiratory motor output at 70.5 ± 9.5 and 70.7 ± 6.3 min, respectively. Likewise, spinal application of DAMGO (0.1 μM; n=3) abolished cervical and thoracic respiratory motor output at 83.4 ± 14.1 and 86.1 ± 17.1 min, respectively. At both concentrations, cervical and thoracic burst amplitudes were reduced by 39-71% after only 30 min of drug application ($p<0.004$ for drug effects; data not shown). Thus, DAMGO

at 0.1 and 1.0 μM could not be used in combination with spinal OGD. Spinal application of DAMGO (0.01 μM; n=4) abolished respiratory motor output before 90 min in only 2/4 preparations, but cervical and thoracic burst amplitudes were reduced by 36 ± 22% and 54 ± 2%, respectively, after 90 min ($p<0.015$ for drug effects; data not shown). DAMGO (0.01 μM) was used for the OGD experiments because this concentration produced the least deleterious effects on respiratory motor output.

V. DISCUSSION

This is the first study to demonstrate that spinal DOR activation before, during, and after spinal OGD onset prolongs spontaneously produced respiratory spinal motor output in neonatal rats *in vitro*. Thus, spinal DOR activation appears to be a versatile mechanism for protecting spinal motor networks during spinal OGD. Our data also suggest that there are complex interactions between opioid and ischemic signaling cascades. Naloxone prolonged spinal motor output during OGD, but end-point times were not altered by spinal MOR activation or DOR activation/MOR blockade, suggesting that spinal MOR activation was not neuroprotective. In contrast, DOR activation (with DPDPE) appears to be a potent neuroprotectant that attenuates the deleterious effects of the ischemic signaling cascade at various time points relative to OGD onset.

Inspiratory spinal motor output is decreased by spinal OGD

The neonatal rat brainstem-spinal cord preparation produces the inspiratory phase of the respiratory rhythm for >2 h *in vitro*, which allows quantification of spontaneous motor output from identified

ventral spinal nerve roots (Wang *et al.*, 1996; Duffin *et al.*, 2003). Only the inspiratory phase was typically produced in this study because the pons was removed from our preparations (Smith *et al.*, 2007). When control aCSF bathed the brainstem and spinal cord, the PO_2 in the superfusate was 458 ± 17 mm Hg. PO_2 levels in superfused brainstem-spinal cord preparations decrease by ~100 mm Hg/100 μm starting at 200 μm above the tissue surface due to unstirred layers of solution (Okada *et al.*, 1993). Thus, respiratory rhythm generating neurons in the brainstem and spinal motoneurons were functioning under aerobic conditions during baseline recordings (Okada *et al.*, 1993; Brockhaus *et al.*, 2003). When hypoxic OGD solution was applied to the spinal cord, PO_2 levels in the spinal compartment rapidly decreased to 165 ± 22 mm Hg within 10 min. This suggests that spinal motoneurons were likely severely hypoxic within 10 min of the switch to OGD solution since PO_2 in brainstem-spinal cord preparations decrease in parallel with the bath PO_2 (Brockhaus *et al.*, 1993).

Motor function is rapidly impaired soon after OGD onset in experimental spinal cord ischemia models. *In vivo* spinal ischemia in swine causes paraplegia and severe paresis 24 h after a 30-min aortic artery clamp (Lee *et al.*, 2008). Similarly, *in vitro* neonatal rat (P6-7) sagittally-hemisected lumbar spinal cords exposed to aglycemic and ischemic solutions have depressed electrically-evoked ventral spinal root potentials within 30-35 min (Jha and Deshpande, 2003; Deshpande and Jha, 2004). Spontaneous respiratory spinal motor activity may be more sensitive to OGD than electrically-evoked potentials because only 2/17 preparations exposed to OGD in our study remained rhythmically active after 30 min. However, differences in bath size, flow rates, animal age, and tissue condition (*i.e.*, hemisected versus intact cord) may account for the difference in OGD sensitivity *in vitro*.

Sustained DOR activation attenuates OGD-induced neuronal dysfunction

Sustained DOR activation before ischemia *in vivo* or OGD *in vitro* decreases neuronal damage and improves markers of normal neuronal function. For example, *in vivo* DOR agonist administration in adult rats 15-45 min before ischemia improves CA1 (Su *et al.*, 2007; Charron *et al.*, 2008) and CA3 neuronal survival (Iwata *et al.*, 2007), increases DOR protein expression (Tian *et al.*, 2008b), reduces brain infarct and neurological deficits (Tian *et al.*, 2008b) as well as improves behavior and motor scores (Su *et al.,* 2007; Charron *et al.*, 2008). Similarly, several DADLE injections administered prior to middle cerebral artery occlusion in rats decreases infarct size and apoptosis (Borlongan *et al.*, 2009). In cortical neuron cultures, sustained DOR activation at the onset of glutamate excitotoxicity or hypoxia preserves membrane integrity, reduces swelling in neuronal bodies, decreases soma vacuolation and neurite fragmentation, and reduces lactate dehydrogenase release 4-24 h after injury (Zhang *et al.*, 2000; Zhang *et al.*, 2002).

In central motor circuits, however, less is known about OGD sensitivity and DOR-dependent neuroprotection. Intrathecal SNC-80 (DOR agonist) administration in adult rats 9-11 min before spinal ischemia improves hindlimb function 48 h later (Horiuchi et al, 2004) and decreases white matter injury in the spinal cord (Horiuchi et al, 2008). In this study, spinal DOR activation prior-to and during spinal OGD increased end-point times by up to 94% (1.0 □M DPDPE, thoracic). The larger DOR-induced end-point times for thoracic motor output may be due to regional differences in DOR expression and intracellular signaling within the spinal cord. Nevertheless, this is the first study to quantify DOR-dependent preservation of spontaneous, continuously active, neonatal spinal motor function during OGD.

DOR activation post-OGD onset attenuates OGD-induced neuronal dysfunction

Ideally, neuroprotective agents would provide neuronal protection after the onset of brain ischemia because therapeutic treatment is often started after clinical signs are manifested. Few studies, however, have specifically tested whether DOR activation after ischemia or OGD onset protects neural networks. For example, DOR activation immediately after transient middle cerebral artery occlusion in adult rodents reduces neurological deficits and infarct volume (Govindaswami *et al.*, 2008; Tian *et al.*, 2008a). To our knowledge, this is the first study to demonstrate DOR-dependent preservation of motor network function after OGD onset in a neonatal mammalian spinal cord *in vitro*. After 15 min of spinal OGD, spinal DOR activation (with ongoing spinal OGD) increased end-point times by up to 80% (1.0 □M DADLE, thoracic). These studies suggest that DOR activation disrupts the deleterious ischemic signaling cascade at multiple steps even if after the cascade is initiated. Further studies will be required to identify the time when respiratory motor output is irreversibly abolished or impaired despite spinal DOR agonist application.

Brief DOR activation before spinal OGD attenuates OGD-induced neuronal dysfunction

Few experiments have tested whether a brief period of DOR activation is protective against a future ischemic or OGD event. Tan-67 (selective DOR agonist) administration to adult rats 24 h before permanent right middle cerebral artery occlusion decreases infarct size and improves neurologic functional outcome (Zhao *et al.*, 2006a). Similarly, under *in vitro* conditions, Tan-67 application to organotypic hippocampal slices 24 h before a 35-min OGD exposure reduces neuronal death in CA1 neurons (Zhao *et al.*, 2006a). In this study, spinal application of DOR agonists 25 min prior to spinal OGD prolonged end-point times by up to 72% (1.0 μM DPDPE, thoracic). Of the two DOR agonists, DPDPE was

more effective at increasing respiratory burst frequency at this concentration (see Figs. 6B-D), but neither DPDPE nor DADLE provided neuroprotection with respect to burst amplitude. Thus, brief spinal DOR activation appeared to induce signaling mechanisms that preserved respiratory burst frequency, but not amplitude. One caveat is that it is not known whether the DOR agonist drugs remained within the spinal tissue during the 25-min washout period and were released slowly to continuously activate spinal DOR receptors. Future experiments would be required to rule out this potential caveat.

Role of spinal opioid receptors during OGD

In the literature, there was controversy as to whether neonatal rats even expressed DOR in the CNS. Although DOR expression changes rapidly during development (Beland and Fitzgerald, 2001; Kivell *et al.*, 2004), DOR are expressed in the neonatal rat forebrain (Milligan *et al.*, 1987; Szucs *et al.*, 1990), brainstem (Kivell *et al.*, 2004) and spinal cord (Attali *et al.*, 1990). Within the spinal cord, functional DOR are expressed at P0 and have similar affinity for specific agonists as adults (Attali *et al.*, 1990). In addition, our data strongly suggest that functional DOR are expressed in the neonatal rat spinal cord because DOR agonists prolong respiratory motor output during spinal OGD.

Although DOR activation can provide neuroprotection, the results of blocking endogenous DOR (or other opioid receptors) before and during OGD are controversial. For example, intraperitoneal injections of naltrindole (DOR antagonist) 30 min prior to forebrain ischemia increase hippocampal CA1 neuronal death (Iwata *et al.*, 2007). Also, in cultured cortical neurons, naltrindole application increases lactate dehydrogenase release during normoxic and hypoxic conditions (Zhang *et al.,* 2002*)* as well as increases sodium azide-induced mitochondrial respiratory chain injury (Zhu *et al.*, 2009). These data are consistent with the hypothesis that endogenous DOR activation is neuroprotective. In contrast, naltrindole

application to hippocampal slices prior to brief OGD exposure improves recovery of population spike amplitude (Ammon-Treiber *et al.*, 2005). In this study, the DPDPE-dependent increase in cervical and thoracic end-point times was blocked by naltrindole, which suggests that DPDPE acted via DOR activation. Since naltrindole alone did not alter the response to spinal OGD, it appears that endogenous spinal DOR activation does not contribute to neuroprotection in these preparations.

Surprisingly, naloxone (general opioid receptor antagonist) increased end-point times by 22% (cervical) and 34% (thoracic) when bath-applied prior-to and during spinal OGD. Since naloxone blocks DOR and MOR, and DADLE can crossover to activate MOR (Goldstein and Naidu, 1986), we hypothesized that endogenous MOR activation was somehow involved in the response to spinal OGD. However, MOR activation alone with spinal OGD did not alter end-point times, and DOR activation/MOR blockade did not act additively (or synergistically) to increase end-point times. These data suggest that there are complex interactions between opioid signaling pathways and the ischemic cascade induced by spinal OGD. On the other hand, naloxone may have exerted a wide range of neuroprotective biological effects that were not related to blocking opioid receptors. For example, naloxone administration following ischemia suppresses cytokine/chemokine production and preserves neuronal proteins (Chen *et al.*, 2001; Liao *et al.*, 2003) as well as restores mitochondrial activities or energy metabolism (Chen *et al.*, 2000). Thus, spinal MOR activation or blockade may play only a minimal role in spinal neuroprotection.

Potential mechanisms of DOR-dependent protection against OGD

Reduced blood flow to the brain impairs O_2 and glucose delivery, and initiates a cascade of events that eventually causes cell death (Pugliese *et al.*, 2003; Bickler, 2004). The inability to generate ATP causes neuronal depolarization, deterioration of Na^+ and K^+ ion homeostasis, and excessive release of

excitatory neurotransmitters. AMPA and NMDA receptor activation increases Na^+ and Ca^{2+} ion influx, which produces further neuronal depolarization, and leads to free radical production. Acute excitotoxicity leads to edema, neuronal damage, and cell death.

Activating endogenous central DOR is an attractive strategy for providing neuroprotection because DOR activation disrupts acute excitotoxic events and signaling pathways at multiple points to preserve ionic homeostasis and cell membrane integrity. For example, DOR activation inhibits excitotoxic influx of Na^+ ions via voltage-gated Na^+ channels (Chao *et al.*, 2008, 2009) and attenuates OGD-induced increases in extracellular K^+ (Chao *et al.*, 2007a,b). DOR activation also acts presynaptically to prevent glutamate release (Ostermeier *et al.*, 2000) and postsynaptically to attenuate Na^+ ion influx via NMDA receptors (Chao *et al.*, 2009). The intracellular signaling pathways for DOR-dependent neuroprotection are not well established. However, in embryonic cortical neuron cultures, DOR activation causes mitogen-activated protein kinase (MAPK) to phosphorylate extracellular signaling-regulated kinase (ERK) and prevent OGD-induced p38 phosphorylation, suggesting that the protective effects of DOR activation may be due to the balance of ERK and p38 activation (Sun *et al.*, 2009). Taken together, these DOR-dependent mechanisms disrupt acute excitotoxicity at multiple points, and ultimately attenuate the cytotoxic rise in intracellular [Ca^{2+}] (Chao *et al.*, 2010).

In this study, detailed mechanisms related to DOR activation were not addressed and beyond the scope of the present work. However, we hypothesize that prolongation of spinal respiratory motor output was primarily due to DOR-dependent attenuation of the early stages of OGD-induced excitotoxicity, such as those described above. DOR-dependent neuroprotection had to be fast-acting because spinal DOR activation prolonged respiratory motor output even 15 min after the onset of spinal OGD. Further studies will be required to test whether DOR-dependent mechanisms of neuroprotection first described in cortical neurons or cell cultures apply to the neonatal spinal cord.

Clinical significance of DOR-dependent neuroprotection

Due to the unpredictability of perinatal ischemic events, candidate neuroprotective agents must rapidly cross the blood-brain and placental barriers, provide long-lasting protection when administered before, during, or after ischemia, and have minimal adverse side effects (Johnson and Turner, 2010). To minimize potential clinically adverse effects, we used peptidergic drugs because some non-peptidergic DOR agonists, such as SNC-80, have been linked to convulsions in rats (Broom *et al.*, 2002b; Jutkiewicz *et al.*, 2005), mice (Comer *et al.*,1993; Broom *et al.*, 2002a), and nonhuman primates (Dykstra *et al.*, 1993; Negus *et al.*, 1994; Pakarinen *et al.*, 1995). Peptidergic DOR agonists may also cause EEG changes, but do not produce overt convulsions (Haffmans and Dzoljic, 1983), and there are examples where DPDPE was used safely without causing convulsions (Torregrossa *et al.*, 2006).

Since opioid-induced respiratory depression is a significant life-threatening side effect, it's important to consider whether selective central DOR activation causes respiratory depression in neonatal mammals. The literature regarding DOR-dependent respiratory depression in adult mammals is controversial, in part, because several studies used relatively non-specific DOR agonists (Shook *et al.*, 1990). Also, studies differ considerably with respect to species, drug dosage, method (bolus vs. infusion) and route (intravenous vs. intracerebroventricular) of drug administration, and animal state (awake vs. anesthetized) (Shook *et al.*, 1990; Johnson and Turner, 2010). However, in neonatal mammals, DOR activation does not appear to cause respiratory depression with the use of highly selective DOR agonists. For example, intraperitoneal DPDPE injections (0.1 mg/kg) do not alter respiratory output in intact neonatal P1 rats (Greer *et al.*, 1995). Also, bath-applied DPDPE does not alter spinal respiratory motor output or bulbospinal respiratory neuronal discharge in neonatal rat brainstem-spinal cord preparations (Greer, 1995; Takita *et al.* 1997; Takeda *et al.*, 2001). In this study, respiratory motor output was not altered by spinal application of DPDPE for >90 min. Thus, it is possible that DOR agonist drugs may be

safely used to treat perinatal ischemia in a wide variety of clinical conditions. In contrast, spinal MOR activation at even very low concentrations reduced spinal respiratory burst amplitude in isolated neonatal rat spinal cords. Therefore, MOR agonist drugs are not good candidates to provide clinically effective neuroprotection against ischemia.

Conclusions

Taken together, our data suggest spinal DOR activation can attenuate or delay the deleterious effects caused by OGD in the neonatal spinal cord. Also, this study shows that DOR activation can protect respiratory spinal motor networks, which are necessary for life. Thus, selective DOR agonist drugs may be ideal for neuroprotection because DOR activation does not cause respiratory depression and provides neuroprotection with great flexibility with respect to the timing of drug administration relative to the ischemic event.

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Figure 1: Neonatal rat brainstem-spinal cord preparation produces spontaneous respiratory motor output. (A) Drawing of a brainstem-spinal cord split-bath preparation showing suction electrodes attached at ventral spinal roots at C4 and T5. Integrated and rectified spinal respiratory motor bursts are shown to the right of the suction electrodes. A plastic barrier at spinal segment C1 separates the brainstem compartment (upper) from the spinal cord compartment (lower). (B) Time-compressed records of spinal respiratory motor bursts are shown for a brainstem-spinal cord preparation bathed in aCSF for 120 min.

Fig. 1

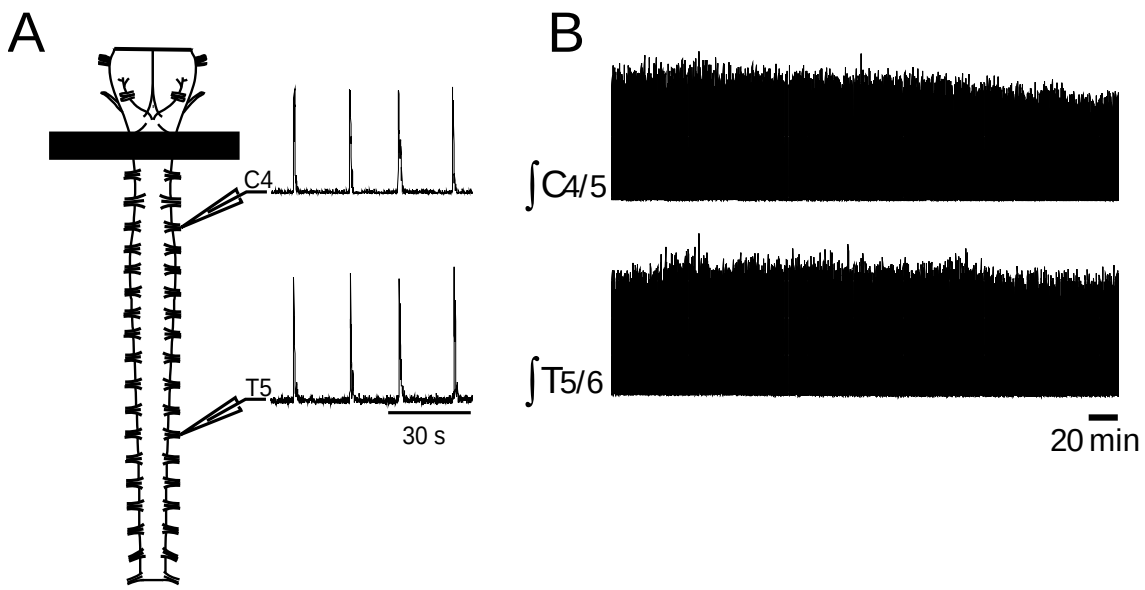


Figure 2: OGD decreases spinal respiratory burst amplitude and frequency. (A) Compressed records of integrated cervical (upper trace) and thoracic (lower trace) spinal respiratory motor output. Horizontal bar over the cervical traces indicates time when OGD solution was applied. Arrow and label shows when respiratory motor output was abolished. (B) Sample traces of thoracic motor output showing relatively little change in burst shape during OGD Traces 1, 2, and 3 were taken from the thoracic traces at the left in (A). (C-D) OGD (open diamonds) decreased cervical (C) and thoracic burst amplitude (D) compared to preparations exposed to aCSF for 120 min (open circles). (E) Normalized average thoracic burst frequency is shown for preparations exposed to aCSF for 120 min (open circles) or OGD (open diamonds). (F) Changes in spinal compartment PO_2 are shown with spinal OGD onset starting at the 5-min time point. Filled symbols indicate significant time-dependent changes from baseline; asterisks indicate significant OGD-induced changes compared to aCSF time controls.

Fig. 2

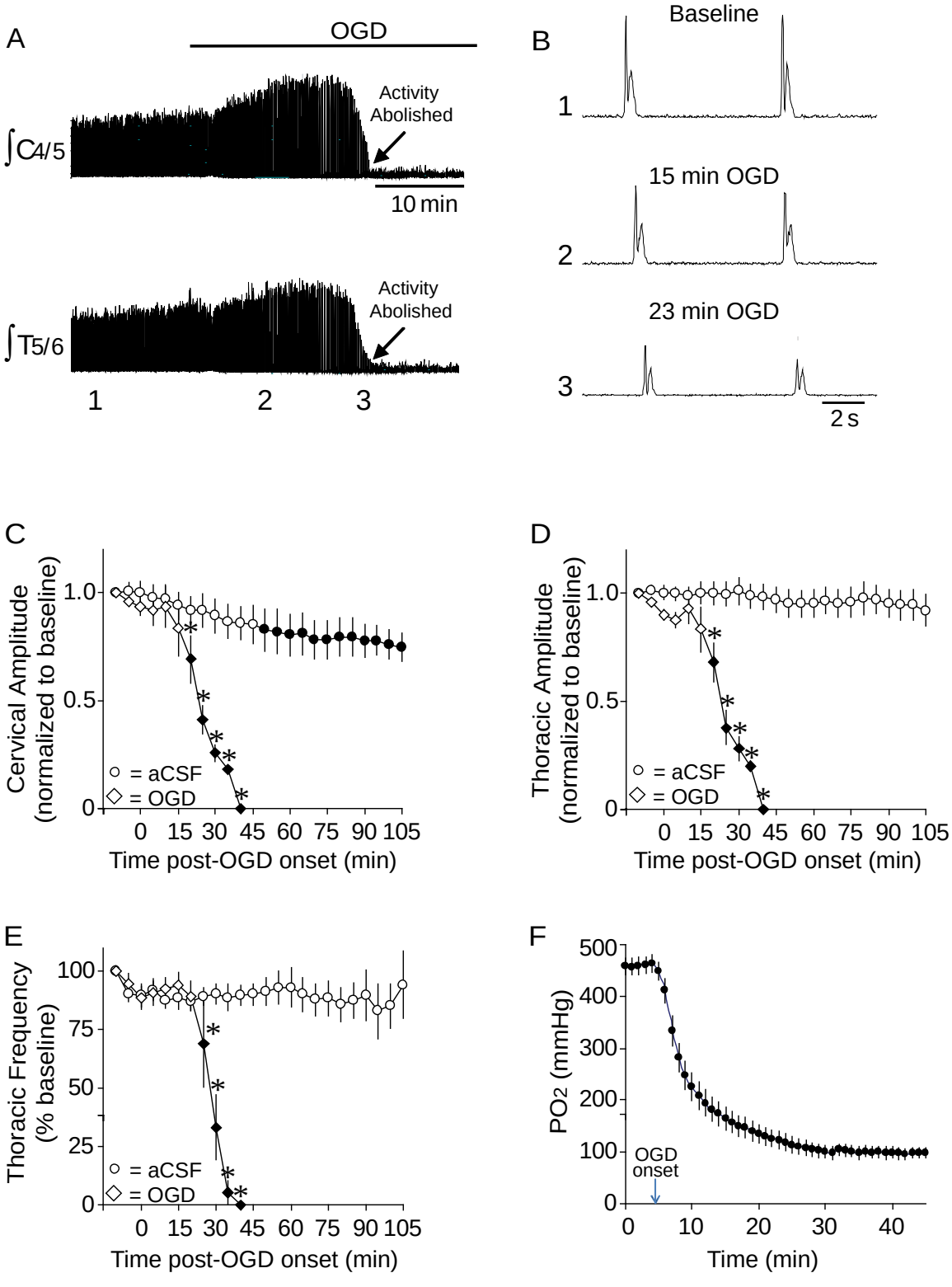

A
OGD
Activity Abolished
∫C4/5
10 min
Activity Abolished
∫T5/6
1
2
3
B
Baseline
1
15 min OGD
2
23 min OGD
3
2 s
C
Cervical Amplitude (normalized to baseline)
1.0
0.5
0
○ = aCSF
◇ = OGD
0 15 30 45 60 75 90 105
Time post-OGD onset (min)
D
Thoracic Amplitude (normalized to baseline)
1.0
0.5
0
○ = aCSF
◇ = OGD
0 15 30 45 60 75 90 105
Time post-OGD onset (min)
E
Thoracic Frequency (% baseline)
100
75
50
25
0
○ = aCSF
◇ = OGD
0 15 30 45 60 75 90 105
Time post-OGD onset (min)
F
PO2 (mmHg)
500
400
300
200
100
0
OGD onset
0 10 20 30 40
Time (min)

Figure 3: DOR activation prior-to and during spinal OGD prolongs respiratory spinal burst frequency. (A) Normalized thoracic burst frequency is shown for preparations exposed to spinal OGD alone (closed diamonds), or spinal OGD with either DADLE at 0.1 μM (open triangles), 1.0 μM (open circles), or 10 μM (open squares) applied prior-to and during OGD. Horizontal lines indicate timing of drug (dotted line) and OGD application (solid line). (B) Normalized thoracic burst frequency is shown for preparations exposed to spinal OGD alone (closed diamonds), or spinal OGD with either DPDPE at 0.1 μM (open triangles), 1.0 μM (open circles), or 10 μM (open squares) applied prior-to and during OGD. Filled symbols indicate significant time-dependent changes from baseline; asterisks indicate significant OGD-induced changes compared to aCSF time controls; daggers indicate significant drug effect.

Fig. 3

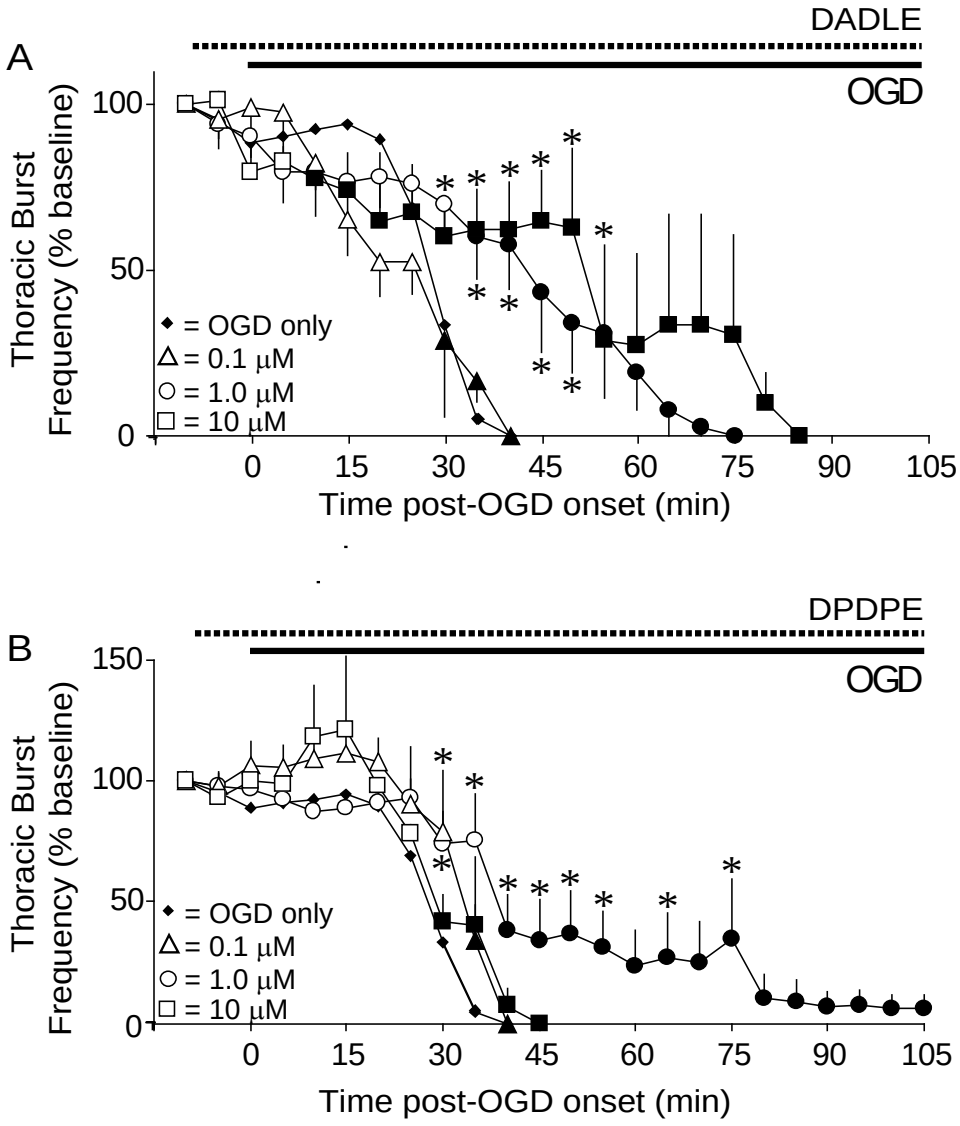

A
DADLE
OGD
Thoracic Burst Frequency (% baseline)
100
50
0
= OGD only
= 0.1 μM
= 1.0 μM
= 10 μM
0
15
30
45
60
75
90
105
Time post-OGD onset (min)
B
DPDPE
OGD
Thoracic Burst Frequency (% baseline)
150
100
50
0
= OGD only
= 0.1 μM
= 1.0 μM
= 10 μM
0
15
30
45
60
75
90
105
Time post-OGD onset (min)

Figure 4: DOR activation prior-to and during spinal OGD increases end-point times and respiratory spinal burst amplitude. (A-B) End-point times (*i.e.*, time from OGD onset to time when spinal nerve motor activity was abolished) were increased by spinal application of DADLE (gray bars) and DPDPE (black bars) for cervical (A) and thoracic (B) respiratory motor output compared to OGD (white bars). (C) DPDPE (1.0 μM, open squares), but not DADLE (1.0 μM, open circles), increased cervical respiratory burst amplitude during spinal OGD. Horizontal lines indicate timing of drug (dotted line) and OGD application (solid line). (D) DPDPE or DADLE increased thoracic respiratory burst amplitude during spinal OGD. Symbols are the same as those in Fig. 3.

Fig. 4

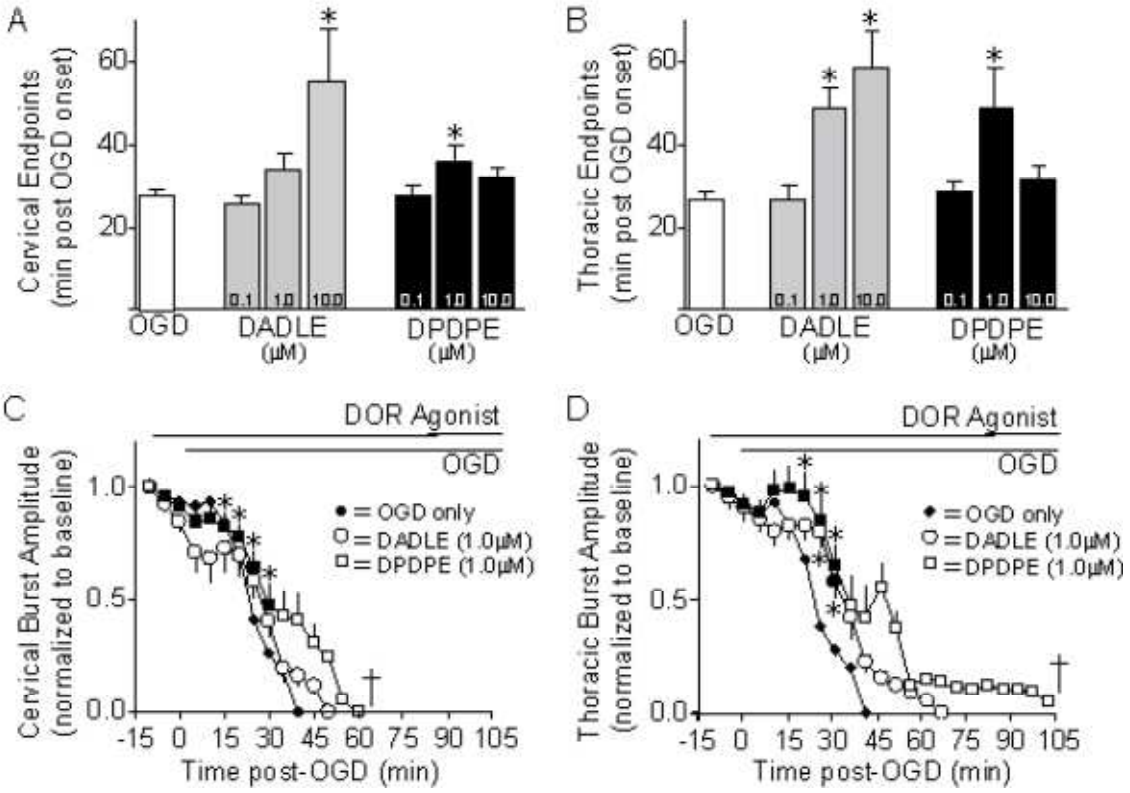

A
Cervical Endpoints
(min post OGD onset)
60
40
20
0.1
1.0
10.0
OGD
DADLE
(μM)
DPDPE
(μM)
B
Thoracic Endpoints
(min post OGD onset)
C
Cervical Burst Amplitude
(normalized to baseline)
DOR Agonist
OGD
1.0
0.5
0.0
= OGD only
= DADLE (1.0μM)
= DPDPE (1.0μM)
-15 0 15 30 45 60 75 90 105
Time post-OGD (min)
D
Thoracic Burst Amplitude
(normalized to baseline)

Figure 5: DOR activation post-OGD onset prevents OGD-induced decrease in respiratory burst frequency and amplitude. (A-B) Thoracic burst frequency was increased over time with 1.0 μM DADLE (A) or 1.0 μM DPDPE (B) (open circles) compared to OGD (closed diamonds) when the drugs were applied 15 min after spinal OGD onset. (C-D) End-point times were increased by DADLE (gray bars) and DPDPE (black bars) for cervical (C) and thoracic (D) respiratory motor output compared to OGD (white bars). (D-E) DPDPE (open squares), but not DADLE (open circles), increased burst amplitude at a few time points during spinal OGD for cervical (E) and thoracic (F) respiratory motor output. Symbols are the same as those in Fig. 3.

Fig. 5

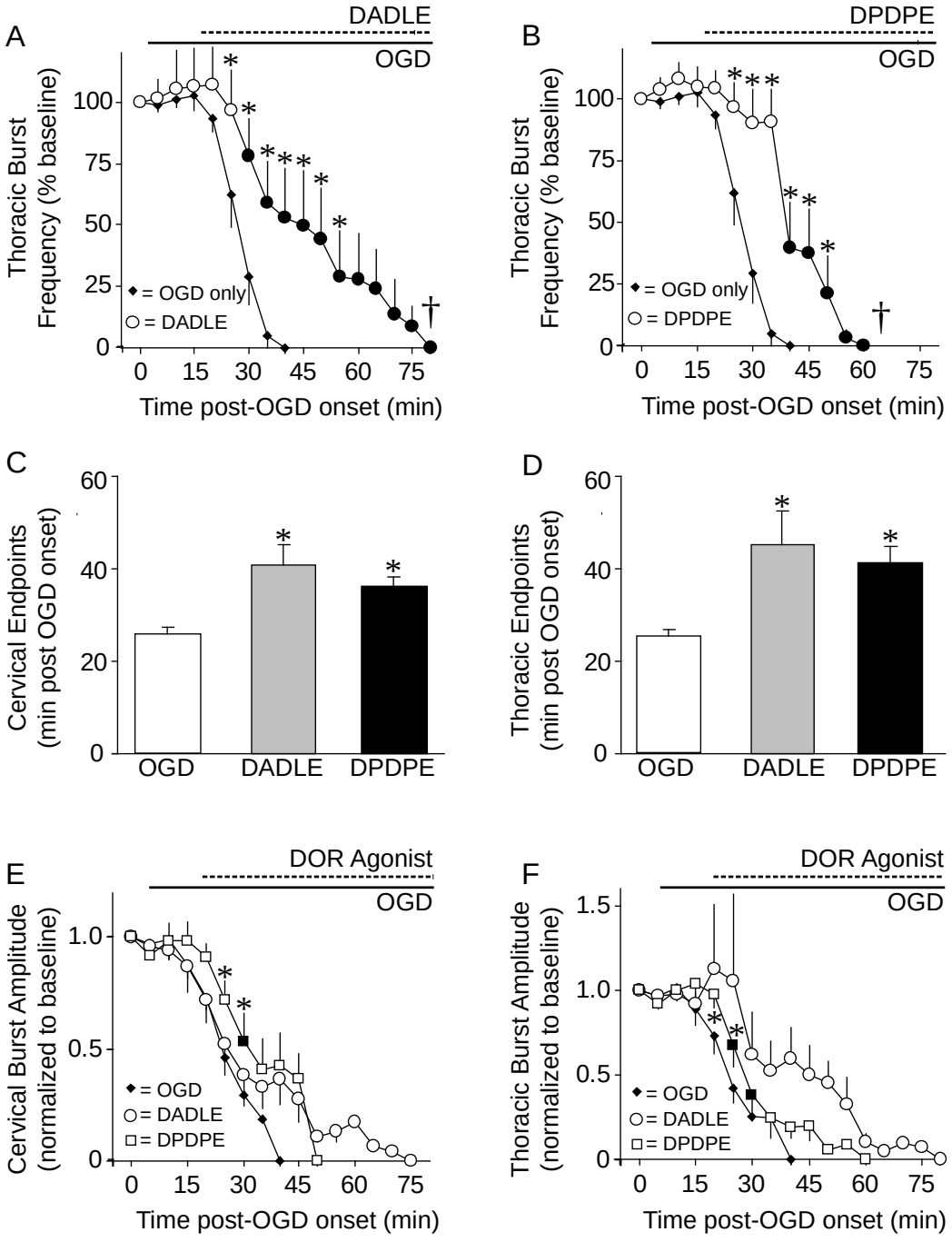

A
DADLE
OGD
Thoracic Burst Frequency (% baseline)
100
75
50
25
0
= OGD only
= DADLE
0 15 30 45 60 75
Time post-OGD onset (min)
B
DPDPE
OGD
Thoracic Burst Frequency (% baseline)
= OGD only
= DPDPE
Time post-OGD onset (min)
C
Cervical Endpoints (min post OGD onset)
60
40
20
0
OGD
DADLE
DPDPE
D
Thoracic Endpoints (min post OGD onset)
E
DOR Agonist
OGD
Cervical Burst Amplitude (normalized to baseline)
1.0
0.5
= OGD
= DADLE
= DPDPE
Time post-OGD onset (min)
F
DOR Agonist
OGD
Thoracic Burst Amplitude (normalized to baseline)
1.5
1.0
0.5
Time post-OGD onset (min)

Figure 6: DOR activation 25 min before spinal OGD prevents OGD-induced decrease in respiratory burst frequency, but not amplitude. (A-B) Thoracic burst frequency was not changed with 1.0 μM DADLE (A), but increased with 1.0 μM DPDPE (B) (open circles) compared to OGD (open diamonds). (C) End-point times were increased by DADLE (gray bar) and DPDPE (black bar) for cervical respiratory motor output compared to OGD (white bar). (D) End-point times were increased by DPDPE (black bar), but not DADLE (gray bar), for thoracic respiratory motor output compared to OGD (white bar). (E-F) DADLE (open circles) and DPDPE (open squares) did not alter cervical (E) or thoracic (F) respiratory burst amplitude compared to OGD (open diamonds). Symbols are the same as those in Fig. 3.

Fig. 6

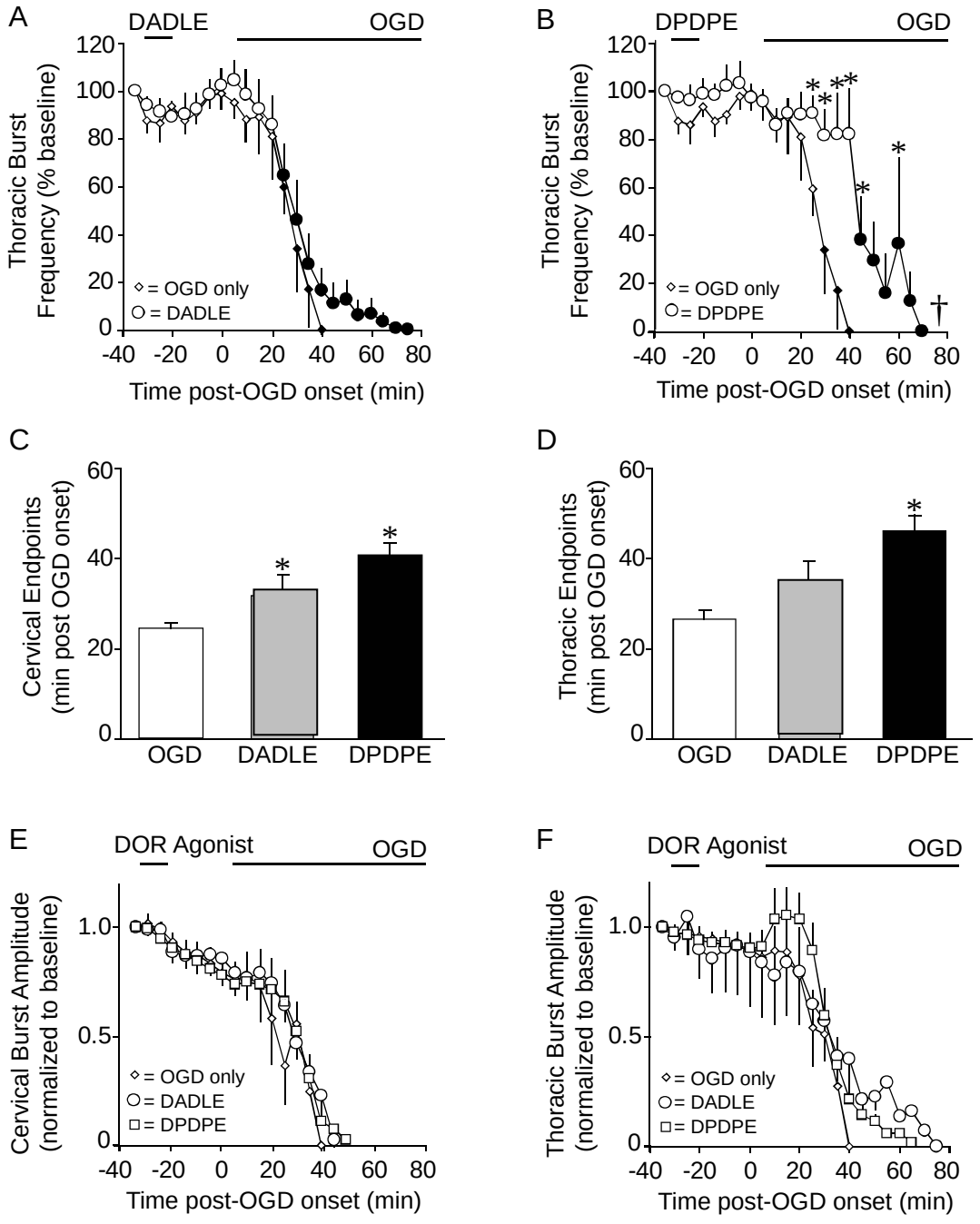

A
DADLE
OGD
Thoracic Burst Frequency (% baseline)
120
100
80
60
40
20
0
◇ = OGD only
○ = DADLE
-40 -20 0 20 40 60 80
Time post-OGD onset (min)
B
DPDPE
OGD
Thoracic Burst Frequency (% baseline)
◇ = OGD only
○ = DPDPE
†
Time post-OGD onset (min)
C
Cervical Endpoints (min post OGD onset)
60
40
20
0
OGD
DADLE
DPDPE
D
Thoracic Endpoints (min post OGD onset)
OGD
DADLE
DPDPE
E
DOR Agonist
OGD
Cervical Burst Amplitude (normalized to baseline)
1.0
0.5
0
◇ = OGD only
○ = DADLE
□ = DPDPE
Time post-OGD onset (min)
F
DOR Agonist
OGD
Thoracic Burst Amplitude (normalized to baseline)
◇ = OGD only
○ = DADLE
□ = DPDPE
Time post-OGD onset (min)

Figure 7: DOR antagonists block DPDPE-induced increase in end-point times. (A-B) For cervical (A) and thoracic (B) respiratory motor output, end-point times for OGD only ("No Drug"; white bar) and 1.0 μM DPDPE application before and during OGD (light gray bar) are shown (data from Figs. 4A, 4B). Naltrindole (10 μM) application before and during DPDPE/OGD application (dark gray bar) blocked DPDPE-dependent prolongation of end-point times. Naltrindole application before and during OGD (black bar) did not alter end-point times compared to OGD only. Asterisks indicate $p<0.05$ compared to OGD.

Fig. 7

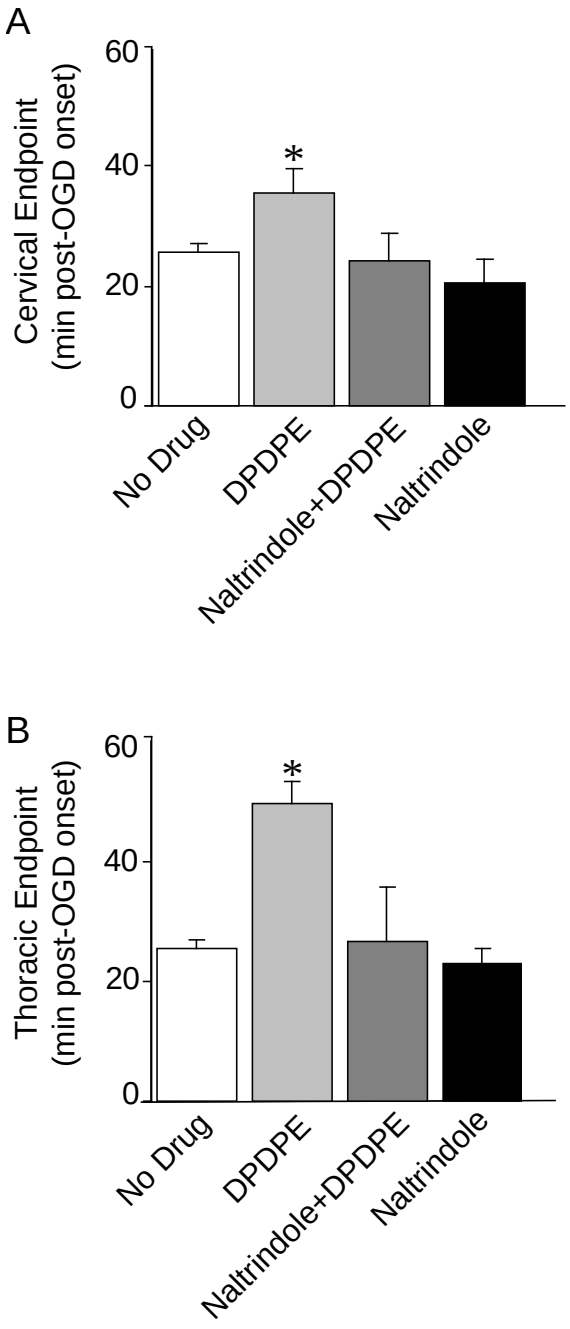
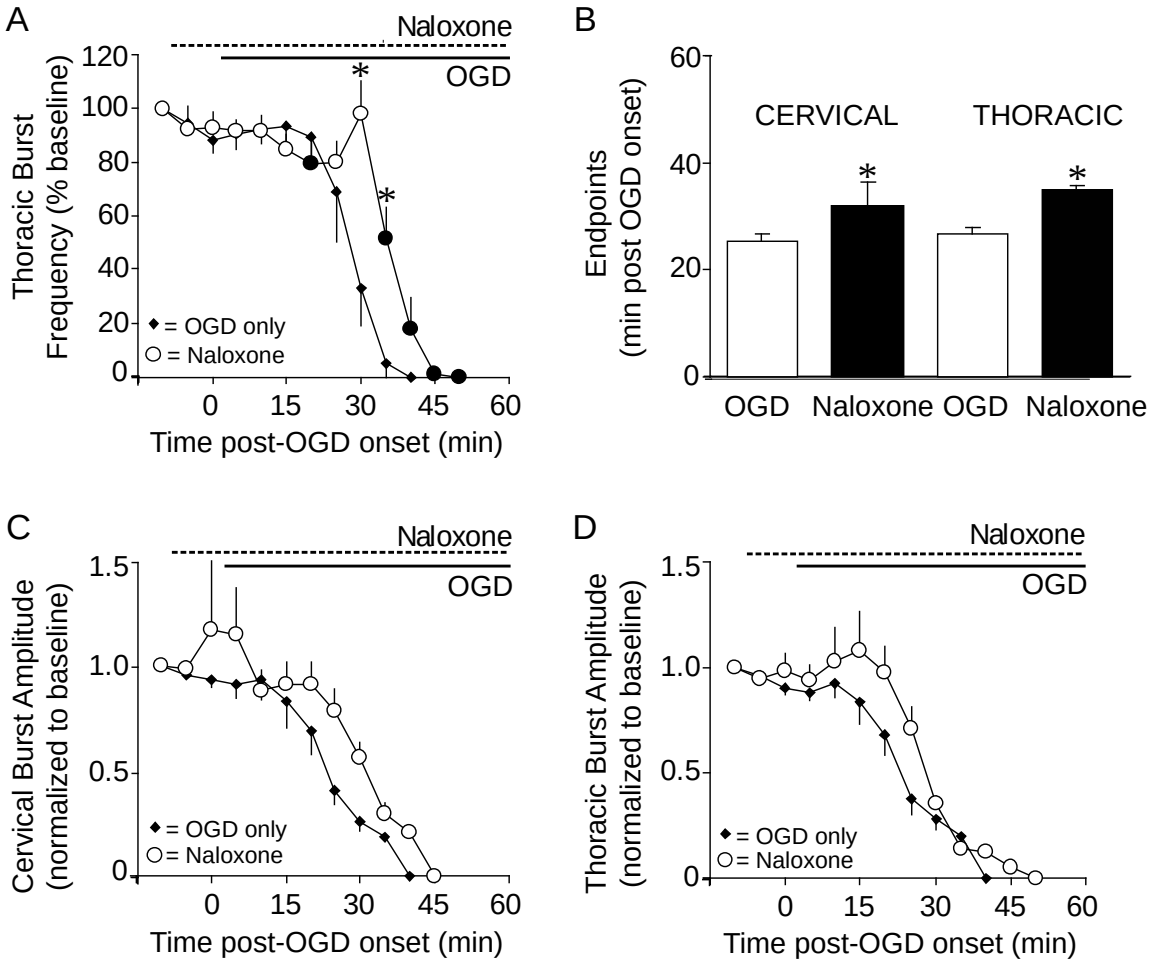

A
Cervical Endpoint
(min post-OGD onset)
60
40
20
0
*
No Drug
DPDPE
Naltrindole+DPDPE
Naltrindole
B
Thoracic Endpoint
(min post-OGD onset)
60
40
20
0
*
No Drug
DPDPE
Naltrindole+DPDPE
Naltrindole

Fig. 8. Naloxone prolongs spinal respiratory output. (A) Naloxone (1.0 □M) applied spinally 10 min before and during spinal OGD (open circles) increased thoracic burst frequency at 30-35 min post-OGD onset (OGD data = filled diamonds). (B) Cervical and thoracic end point times were increased by naloxone (black bars) compared to OGD (white bars); asterisks indicate $p<0.05$ compared to OGD. (C, D) Naloxone (open circles) did not alter cervical (C) or thoracic (D) burst amplitude compared to OGD (filled diamonds). Symbols are the same as in Fig. 3.

Fig. 8


A
Naloxone
OGD
Thoracic Burst Frequency (% baseline)
120
100
80
60
40
20
0
= OGD only
= Naloxone
0
15
30
45
60
Time post-OGD onset (min)
B
Endpoints (min post OGD onset)
60
40
20
0
CERVICAL
THORACIC
OGD
Naloxone
OGD
Naloxone
C
Naloxone
OGD
Cervical Burst Amplitude (normalized to baseline)
1.5
1.0
0.5
0
= OGD only
= Naloxone
0
15
30
45
60
Time post-OGD onset (min)
D
Naloxone
OGD
Thoracic Burst Amplitude (normalized to baseline)
1.5
1.0
0.5
0
= OGD only
= Naloxone
0
15
30
45
60
Time post-OGD onset (min)

Chapter 5:

Discussion

Breathing is a basic requirement for life, yet, it is uniquely challenging to provide adequate neuroprotection to the respiratory control system. The rhythm and pattern of breathing is coordinated with a complex network of feedback mechanisms that balance inhibitory and excitatory neuronal signaling. Thus, the respiratory control system stabilizes blood gases, but can also mount a robust ventilatory response to acute challenges (*e.g.*, hypoxia) or compensate for long-term changes in neuronal excitability (*e.g.*, pregnancy). While respiratory-related neurons have a wide range of heterogeneous functions including rhythm generation, pattern formation, chemosensitivity, sensory integration, and muscle innervation, all components are required for the system to function properly. Therefore, an ideal neuroprotective strategy would maintain respiratory network function and protect all components of the system. Enhancing endogenous protective mechanisms is a compelling strategy for developing novel therapies and treatments to protect neuronal function against physiological and pathological conditions because these strategies may be more likely to provide versatile protection to a system with heterogeneous neuronal functions. We studied two separate endogenous protective mechanisms to compensate for challenges in maintaining respiratory neuron function (Fig. 1): (1) $GABA_AR$ subunit changes to protect against potential excessive neuronal inhibition potentially caused by elevated brain neurosteroid levels and (2) delta opioid receptor (DOR) activation to protect neuronal function during excitotoxicity in an *in vitro* model of spinal neonatal stroke.

I. How might the respiratory control system adjust for increased neuronal inhibition?

Increased $GABA_AR$ epsilon subunit incorporation in respiratory-related brainstem regions

Dynamically changing $GABA_AR$ subunit composition appears to adjust neuronal excitability during times of increased neuronal inhibition (*e.g.*, pregnancy or the critical period in respiratory control development). Specifically, the expression of the epsilon subunit increases in both PBC-region (Hengen

et al., 2012) and XII motoneurons (Chapter 2) during pregnancy when neuronal inhibition presumably increases due to a 3-fold increase in central allopregnanolone levels (Concas *et al.*, 1998). Paradoxically, brain allopregnanolone levels also increase 3-4 fold during the critical period in respiratory control development (Grobin and Morrow, 2001; Wong-Riley and Liu, 2008), but PBC-region neurons only increase epsilon subunit incorporation for one day and XII motoneurons appear to decrease epsilon subunit incorporation (Chapter 3). This suggests that allopregnanolone may be sufficient but not necessary to increase epsilon subunit expression. These data, along with other recent studies (Hengen *et al.*, 2009, 2011, 2012), suggest that adjusting epsilon subunit expression contributes maintenance of respiratory-related neuronal function when neuronal inhibition is increased.

Potential mechanisms of increased epsilon subunit expression: We hypothesized that increased central allopregnanolone levels led into increased $GABA_AR$ epsilon subunit expression in respiratory-related brain regions. Allopregnanolone increases chloride ion influx through $GABA_AR$ and augments GABAergic signaling. Therefore, elevated central allopregnanolone concentrations could increase intracellular chloride concentrations and hyperpolarize neurons. Interestingly, intracellular chloride concentrations regulate alpha-1, alpha-3, and delta $GABA_AR$ subunit expression in primary cerebellar neurons (Succol *et al.*, 2012). Perhaps, intracellular chloride concentrations also regulate epsilon subunit expression. The increase in brain allopregnanolone levels during pregnancy may change intracellular chloride concentrations and increase epsilon subunit expression in respiratory-related neurons. Potentially, rapidly changing intracellular chloride concentrations could also explain why PBC-region, XII, and NTS neurons had significant differences in epsilon subunit expression in an age- and medullary region-dependent manner during the critical period in respiratory control (Chapter 3).

While the underlying mechanisms controlling epsilon subunit expression are largely unknown, epsilon subunit incorporation in $GABA_ARs$ tends to change during times of physiological stress such as during hibernation, pregnancy, and critical developmental periods. Thus, these findings suggest that

modulating epsilon subunit incorporation is compensatory mechanism that is conserved across development and respiratory-related brain regions. Changing $GABA_AR$ subunit composition to maintain appropriate neuronal excitability is similar to previous descriptions of homeostatic plasticity of neuronal networks, whereby neurons alter GABA and glutamate receptor expression in a compensatory manner in response to excitatory or inhibitory stimuli (Maffei and Turrigiano, 2008; Gainey *et al.*, 2009). Further, the transcription factors CREB and SRF, which are necessary for many types of activity-dependent plasticity, have highly conserved binding sites on the gene encoding the epsilon subunit (West *et al.*, 2001; Pintchovski *et al.*, 2009; Gao *et al.*, 2010). Thus, CREB and SRF have been hypothesized to suppress epsilon subunit transcription until there is a period of reduced neuronal activity (Hengen *et al.*, 2012). Thus, adjusting epsilon subunit incorporation in $GABA_ARs$ may be a novel form of homeostatic plasticity used by the respiratory control system when inhibitory neuronal signaling is increased.

II. Increased epsilon expression: "good" or "bad" for breathing and neuroprotection?

Increased epsilon subunit expression is advantageous for neuroprotection. Epsilon subunit expression is hypothesized (Hengen *et al.*, 2012, Chapters 2, 3, and Appendix) to be linked to increased brain allopregnanolone concentrations that protect neurons from hypoxic and other pathological conditions. However, increasing allopregnanolone levels also increases the risk of excessive neuronal inhibition. Epsilon subunit incorporation confers allopregnanolone-insensitivity, and thus, appears to be a secondary endogenous neuroprotective mechanism that allows respiratory-related neurons to continue to function while brain allopregnanolone levels increase. From a biological perspective, it is logical to preserve breathing while allowing other neurons (*e.g.*, cortical) to risk becoming excessively inhibited during hypoxia and ischemia.

Do brain regions have differential thresholds for increasing epsilon subunit expression?

While epsilon subunit mRNA was found in various respiratory-related nuclei (Chapter 2) and increases in the medulla during the perinatal period (Pape *et al.*, 2009), the PBC appears to have the lowest threshold for increasing epsilon subunit expression during physiological conditions or following drug injection. For example, during the interbout arousal phase of hibernation, PBC-region neurons are resistant to pentobarbital and have increased epsilon subunit protein compared to the cortex (Hengen *et al.*, 2009, 2011). NTS neurons also increase pentobarbital resistance and epsilon subunit protein, but to a lesser extent compared to PBC-region neurons (Hengen *et al.*, 2009, 2011). Similarly, medroxyprogesterone and allopregnanolone injections were sufficient to increase epsilon subunit expression in PBC-region neurons but not XII, NTS, or cortical neurons (Appendix). The threshold for increasing epsilon subunit expression could be related to the importance of the physiological function for maintaining life. For example, if cortical neurons become excessively inhibited, the animal may just simply become drowsy. On the other hand, if PBC neurons are excessively inhibited, inspiratory rhythm generation could fail leading to cessation of breathing.

Is epsilon neuroprotective against ischemia or hypoxia?

The strategy to pharmaceutically activate $GABA_A$Rs as an acute stroke treatment is currently in clinical trials (Liu and Wang, 2013), but the subunits involved in $GABA_A$R-dependent neuroprotection are not well understood. It is likely that epsilon subunit incorporation is neuroprotective against hypoxia and ischemia beyond maintaining neuronal function when brain allopregnanolone levels are increased. For example, during acute excitotoxicity there is increased excitatory drive (*e.g.*, NMDA receptor overactivation, see Fig. 3 in Chapter 1). Epsilon-containing $GABA_A$R have a GABA-independent, constitutive, tonic current that could potentially shunt a portion of the excitatory drive and thereby protect neurons from excitotoxic damage. However, the time-course of increasing epsilon subunit

expression following a rapid increase in brain allopregnanolone levels is unknown. Further, it is also unclear whether epsilon containing $GABA_AR$ in native receptors express a tonic inhibitory current or if the magnitude of this current is large enough to significantly change neuronal excitability. Thus, future studies are needed to determine whether increased epsilon subunit incorporation significantly maintains neuronal excitability under ischemic/excitotoxic conditions.

Can increased epsilon subunit incorporation in $GABA_ARs$ be deleterious?

The possibility exists that increased epsilon subunit expression may be deleterious. Epsilon subunit expression increases during conditions that are physiologically stressful such as pregnancy. However, an important distinction is that not all neurons increase epsilon subunit expression. For example, in XII motoneurons only 8% of neurons from male rats were pentobarbital-resistant, but during pregnancy, resistance increased to 40% of XII motoneurons (Chapter 2). Similarly, using semi-quantitative immunohistochemistry, the amount of epsilon subunit protein increases per PBC-region neuron, however, the total number of epsilon-expressing neurons does not change (Hengen *et al.*, 2012). Thus, not all neurons express epsilon subunits or become resistant to positive allosteric modulation during pregnancy.

Potentially, the ratio of epsilon-expressing and non-epsilon-expressing neurons is determined by the balance between resistance to positive allosteric modulation and the constitutive GABA-independent tonic current that epsilon subunit incorporation confers to $GABA_ARs$. In this speculative model, the net benefit of epsilon subunit expression is bell-curved rather than linear, such that over-expression of epsilon subunits is deleterious. Perhaps, the epsilon subunit incorporation levels achieved during physiological stress (*e.g.*, hibernation, pregnancy, critical periods in development) are carefully optimized to maintain balance between phasic and tonic inhibition in individual neurons and the network. Then, pathological conditions (*e.g.*, hypoxia, ischemia, infection) could stimulate massive

increases in brain allopregnanolone concentrations leading to overexpression of epsilon subunits. For example, inflammation caused by lipopolysaccharide increases brain allopregnanolone levels 2-3 fold (Billiards *et al.*, 2006) and our preliminary data suggest that only one lipopolysaccharide injection (1.0 mg/kg) is sufficient to increase epsilon subunit incorporation. These data suggest that epsilon subunit expression increases during infection. Interestingly in human infants, increased lethargy and respiratory apneas are early signs of infection. We speculate a contributing factor is that brain allopregnanolone levels have increased to an extent that led to epsilon subunit overexpression. Overexpression of epsilon subunits in PBC neurons could create tonic inhibition sufficient to shunt excitatory current in the dendrites, decreasing the inspiratory drive potential, and contributing to central apneas. Epsilon subunit overexpression may also contribute to apnea of prematurity because of the repeated hypoxic episodes that may increase central allopregnanolone levels (Billiards *et al.*, 2006). Therefore, future studies to determine the magnitude and physiological impacts of epsilon subunit-dependent tonic inhibition will be compelling for directing the clinical applications of this research. Overall, increased epsilon subunit incorporation appears to play a protective role for breathing, although the possibility remains that epsilon subunit overexpression may negatively impact respiratory function.

III. Protecting the respiratory control system from excitotoxicity

Activate DOR: One compelling strategy to for protecting respiratory motor output is to activate DOR in ischemia-susceptible animals during the acute excitotoxicity. DOR are highly expressed in extremophile vertebrates such as in hypoxia-resistant turtles and hibernating mammals, and thus are hypothesized to provide neuroprotection (for review see Chao and Xia, 2010). There are many advantages to activating DOR to provide neuroprotection from a clinical perspective. First, DOR drugs

are pleiotropic by disrupting several steps during acute excitotoxicity and attenuating signaling pathways that continue for hours to days after the initial ischemic event. Another important feature is that DOR activation is neuroprotective in a variety of CNS regions including the cortex, hippocampus, and spinal cord, as well as other organs such as the heart (Huang *et al.*, 2007), kidney (He *et al.*, 2013) and intestine (Tubbs *et al.*, 2002). Finally, DOR activation protects neuronal function during ischemia regardless of whether DOR drug administration begins prior-to, during or after the onset of ischemia (Chapter 4). This is an advantage because of the unpredictability in ischemic events and the variability in the duration from ischemic injury to treatment onset in clinical practice.

IV. Ischemic neuroprotection: is there an upper limit for neuroprotection?

Ischemia causes multifactorial damage with an acute phase (excitotoxicity) and a long-lasting phase (inflammation and cell death). Neuroprotective agents with the best clinical potential will attenuate the acute phase of excitotoxic damage but will also decrease damage over a longer time frame (days). Potentially, endogenous neuroprotective mechanisms could be enhanced to attenuate acute excitotoxicity and disrupt the ischemic signaling cascade, altering gene expression to decrease or eliminate inflammation and programmed cell death in the days following injury. While such a mechanism could significantly improve neuronal function following injury, it is unlikely that enhancing a single mechanism will completely eliminate ischemic injury. Pharmaceutically enhancing neuroprotective mechanisms is a compelling strategy to improve outcomes, however, there is likely to be an upper limit to the benefits of enhancing a protective mechanism. For example, increased brain allopregnanolone levels are neuroprotective, but, excessively increasing allopregnanolone concentrations could lead to loss of consciousness (Reddy and Zeng, 2007) or respiratory difficulties. With respect to DOR-dependent neuroprotection, increasing the concentration of the DOR agonist,

DPDPE (see Chapter 4) from 1.0 μM to 10 μM did not further prolong endpoint times of respiratory motor output during OGD. Thus, both neuroprotective strategies we studied appear to have upper limits to the neuroprotective benefits provided.

Why is there naturally so much redundancy in neuroprotection?

From an evolutionary perspective, it is logical to have many overlapping neuroprotective mechanisms that act synergistically to provide comprehensive protection during severe insults (*e.g.*, ischemia) to the CNS. Endogenous neuroprotective mechanisms have common signaling pathways that induce compensatory changes and produce of downstream effects within neuronal networks. Many mechanisms exist to attenuate ischemic damage through similar goals, such as preserving ionic homeostasis and reducing inflammation. Perhaps, there is naturally so much overlap in neuroprotection to address the multilayered effects of ischemic damage and to accommodate for the upper limits of single mechanisms. Further, these overlapping mechanisms may lend flexibility to the neuroprotective response to ischemia. Endogenous neuroprotective mechanisms can be activated under a variety of conditions with flexible timing relative to the ischemic injury. The wide-ranging effects of activating these pathways likely optimize the balance of neuronal function during ischemia and increased neuronal survival post-ischemia.

Neuronal susceptibility to ischemia varies greatly by brain region, so redundancy in protective mechanisms may also exist to account for the heterogeneity in neuronal populations. Since the respiratory control system contains neurons with many subtypes and functions it is possible that endogenous neuroprotective mechanisms may have varying degrees of efficacy based on the specific neuronal subtype. Our data support this hypothesis by demonstrating differential expression of compensatory mechanisms in a region-specific manner (Chapters 2 & 3) and different levels of neuroprotection between respiratory spinal motoneuron pools (Chapter 4). On the other hand, it is

possible that the best strategy for providing neuroprotection to ischemia-susceptible mammals is still evolving and the redundancy is due to ongoing natural selection.

Are expectations for ischemic neuroprotection unrealistic?

The "magic bullet" for neuroprotection is expected to be a single treatment that can be administered at any point in the ischemic signaling cascade to completely restore neuronal function. This achievement has been elusive, due, in large part, to the redundancy and lack of specificity in endogenous neuroprotective mechanisms. For example, many mechanisms may be activated by various pathological stimuli (ischemia, hypoxia, inflammation, drugs) at different time points and once activated, initiate complex signaling cascades with long-lasting effects. Enhancing protective mechanisms should change the course of signaling pathways during ischemia and elicit downstream effects that also protect neuronal function.

Perhaps, greater success in clinical trials will be achieved by shifting research strategies from individual, step-by-step signaling cascades that measure outcomes based on neuronal death or infarct size to wide-ranging strategies that will activate or attenuate many signaling pathways simultaneously and improve functional outcomes. One compelling strategy is to study endogenous protective pathways in extremophile vertebrates because the pathways to provide ischemia resistance have already evolved and are finely-tuned to protect neurons from decreased oxygen or blood flow. Thus, these animals have already solved big clinical problems such as ischemia-reperfusion injury. An important key to future research will be to not only identify individual neuroprotective mechanisms, but to study how these mechanisms function synergistically to optimize neuroprotection. Focusing on eliciting long-lasting neuroprotection through synergistic overlapping network effects that maintain network function across many CNS regions is required for satisfactory clinical outcomes. Candidate mechanisms will induce other protective effects (*e.g.*, allopregnanolone induces epsilon subunits) and induce protection from ischemia

globally (*e.g.*, DOR activation protects throughout the CNS and a variety of organ systems). New pharmaceutical treatments for ischemia should to pharmacologically enhance many endogenous mechanisms at once to maintain balance between multiple mechanisms (as occurs naturally) and maintain or restore neuronal network function. Also, aligning experimental procedures with clinical goals (*e.g.*, experimentally start treatments post-ischemia onset and measure functional outcomes) is likely to improve clinical outcomes for neuroprotection. Hopefully, combining and implementing these research strategies will significantly improve clinical outcomes of ischemic injury in future years.

Fig. 1

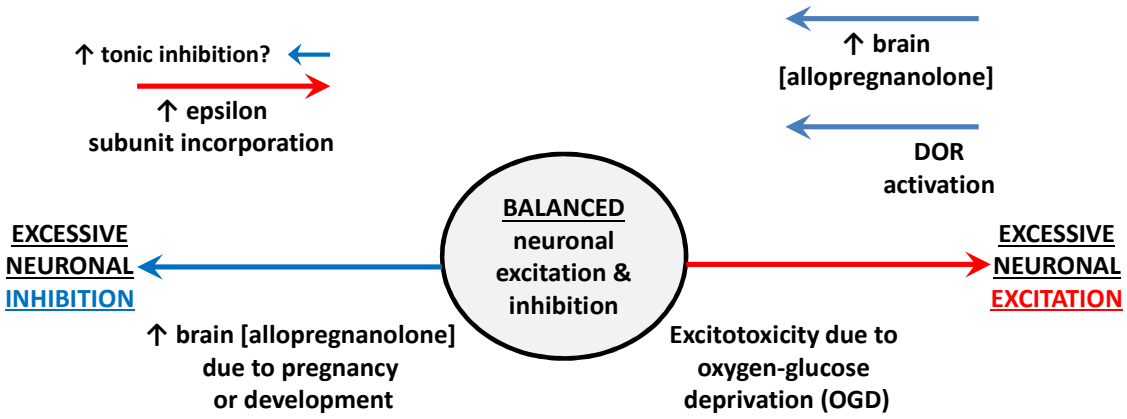

↑ tonic inhibition?
↑ epsilon subunit incorporation
↑ brain [allopregnanolone]
DOR activation
BALANCED neuronal excitation & inhibition
EXCESSIVE NEURONAL INHIBITION
EXCESSIVE NEURONAL EXCITATION
↑ brain [allopregnanolone] due to pregnancy or development
Excitotoxicity due to oxygen-glucose deprivation (OGD)

Figure 1: Model of potential strategies to balance neuronal excitability. The diagram shows optimal balance of excitation and inhibition in the center circle. Physiological or pathological conditions shift the balance towards inhibition (leftward blue arrows) or excitation (rightward red arrows). Compensatory mechanisms involving $GABA_AR$ epsilon subunits or DOR activation act to offset the major shifts towards excessive inhibition or excitation. For example, increased brain allopregnanolone concentrations (during pregnancy or development) threaten neuronal excitability by increasing the risk of excessive neuronal inhibition (leftward blue arrow). We hypothesize that there is an increase in epsilon subunit containing $GABA_ARs$ to confer resistance to allopregnanolone-dependent increases in inhibition and increase excitation (rightward red arrow). One potential drawback to this strategy is that epsilon subunit containing $GABA_ARs$ also mediate a spontaneous tonic inhibition that may have some hyperpolarizing effect (not yet demonstrated in native receptors; small leftward blue arrow). On the other hand, excessive excitation due to oxygen-glucose deprivation (rightward red arrow) is also a threat to neuronal function. Both DOR activation and increased brain allopregnanolone levels attenuate excitotoxicity caused by oxygen-glucose deprivation (leftward blue arrows).

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Appendix:

Medroxy-progesterone acetate (MPA) and allopregnanolone administration are sufficient to increase pentobarbital resistance in Pre-Bötzinger Complex (PBC)-region neurons.

Sara M.F. Turner and Stephen M. Johnson

I. ABSTRACT:

PBC neurons are critical for inspiratory rhythm generation and naturally increase incorporation of the rare $GABA_AR$ epsilon subunit during pregnancy. The $GABA_AR$ epsilon subunit confers resistance to positive allosteric modulators, which typically enhance chloride ion influx in $GABA_ARs$ including allopregnanolone (increased during pregnancy) and pentobarbital. We hypothesize epsilon subunit incorporation in $GABA_ARS$ increases to prevent excessive neuronal inhibition when central neurosteroid concentrations are increased. Thus, we tested whether medroxy-progesterone acetate treatment (MPA, progesterone analogue, 1.0 mg/day, SQ x 5-7 days) or allopregnanolone treatment (1.0 mg/day, SQ x 2 days) is sufficient to increase epsilon subunit incorporation in $GABA_ARs$ in PBC-region neurons. Multichannel electrodes recorded spontaneous action potentials from PBC-region neurons in medullary slices *in vitro* taken from P30 untreated (n=7), oil-treated vehicle control (n=6), MPA-treated (n=4), or allopregnanolone-treated (n=3) rats. To test for epsilon subunits, slices were sequentially exposed to 200 and 300 μM pentobarbital (45 min each). During 300 μM pentobarbital, PBC-region neuron firing rates decreased in control rats to 44±5% (n=68 cells) of baseline and only 13% of cells were pentobarbital resistant (firing rate >80% of baseline during the last 15 min of pentobarbital application). Similarly, in vehicle control rats, pentobarbital application decreased neuronal firing rates to 48±9% of baseline (n=35 cells) and 16% of cells are pentobarbital resistant. In contrast, PBC-region neurons from MPA-treated rats maintained near-baseline values (91±12%; n=31 cells; $p<0.001$) and 52% of cells were pentobarbital resistant. Further, in allopregnanolone treated rats, normalized average firing rates were 138±37% and 44% of PBC-region neurons were pentobarbital-resistant. These data suggest pharmaceutically increased central neurosteroid concentrations increase epsilon subunit incorporation to provide resistance to $GABA_AR$ positive allosteric modulation.

II. INTRODUCTION:

GABAergic signaling is critical for proper respiratory control. $GABA_A$Rs mediate two types of neuronal inhibition: fast, phasic, synaptic inhibition and a constant tonic inhibitory current. In rhythm generating PBC neurons, $GABA_A$Rs modulate neuronal excitability (Shao and Feldman, 1997). For example, tonic GABAergic input constrains the control- and reflexively-induced activities of medullary ventral respiratory column neurons to about 35-50% of the discharge rate without this inhibitory input (Zuperku and McCrimmon, 2002). Thus, excessive GABAergic inhibition in respiratory neurons may diminish respiratory drive, cause apneas, and disrupt blood-gas homeostasis. Compensatory mechanisms within the respiratory control system for increased neuronal inhibition are largely unknown.

Allopregnanolone is a progesterone metabolite that enhances chloride ion influx into $GABA_A$Rs (Bayliss *et al.*, 1992); thereby increasing the risk for excessive neuronal inhibition. Brain allopregnanolone concentrations naturally increase 3-fold during pregnancy (Concas *et al.*, 1998) and 3-4 fold during the critical window in respiratory control development from postnatal days 10-14 (P10-14; Grobin and Morrow, 2001). Our *working hypothesis is that increased central allopregnanolone concentrations induce epsilon subunit incorporation in $GABA_A$Rs of PBC-region neurons to protect breathing from increased neuronal inhibition.* When compared to standard synaptic $GABA_A$Rs (alpha-1, beta-2, alpha-2, beta-2, gamma-2), epsilon-containing $GABA_A$Rs are resistant to positive allosteric modulators such as allopregnanolone and pentobarbital (Irnaten *et al.*, 2002; Wagner *et al.*, 2005). Therefore, increasing epsilon subunit expression may provide resistance to allopregnanolone-dependent increased neuronal inhibition. Neurons in the PBC-region and hypoglossal (XII) motor nucleus increase resistance to pentobarbital during late pregnancy, which suggests increased epsilon subunit containing $GABA_A$RS (Hengen *et al.*, 2012, Chapter 2). Additionally, PBC-region neurons from pregnant rats have

increased epsilon subunit immunoreactivity compared to male or virgin female rats (Hengen *et al.*, 2012). Epsilon subunit incorporation also appears to change in PBC-region, XII, and nucleus tractus solitarius (NTS) neurons during the critical window in respiratory control development (Chapter 3). However, the mechanisms underlying increased epsilon subunit-containing $GABA_AR$ are unknown. We hypothesize that epsilon subunit expression can be pharmacologically induced by chemically mimicking pregnancy with MPA administration and with allopregnanolone injections.

To address these questions, we treated pre-pubescent rats (starting at P23; to eliminate puberty or estrus cycle influences) with medroxy-progesterone acetate (MPA) for 5-7 days. MPA is a progesterone analogue with high affinity for progesterone receptors that also increases brain allopregnanolone levels (Bernardi *et al.*, 2006). Therefore, increased epsilon subunit expression could occur by progesterone receptor activation or by increased brain allopregnanolone concentrations. To test whether increased brain allopregnanolone concentrations are sufficient to increase epsilon subunit incorporation in $GABA_ARs$, rats were injected with allopregnanolone for two days. Following the injections, neuronal sensitivity to pentobarbital was tested *in vitro* in brain slices containing the PBC, XII nucleus, NTS, and cortex. Pentobarbital-resistant neurons were interpreted as putatively expressing epsilon subunits based on previously published results (Hengen *et al.*, 2009, 2011, 2012). Our data show increased pentobarbital-resistance in PBC-region neurons following both MPA- and allopregnanolone-treatment. These results suggest that MPA and allopregnanolone injections are sufficient to induce epsilon subunit-containing $GABA_ARs$ in PBC-region neurons.

III. METHODS

Young rats were divided into 4 groups: untreated, vehicle control (0.2 ml sesame oil), MPA-treated (1.0 mg dissolved in 0.2 ml sesame oil) or allopregnanolone-treated (1.0 mg). Beginning at P23 rats were injected SQ daily for 7 days for MPA-treatment or starting at P26-P28 for 2 days for allopregnanolone-treatment. Medullary and cortical slices (400 μm thick) were taken from P28-P30 treated and untreated rats. Slices were subfused with artificial cerebrospinal fluid (7 mM KCl) and maintained at 37°C for 4 h. Extracellular multichannel electrodes recorded spontaneous action potentials in PBC-region neurons, XII motoneurons, NTS neurons, and cortical neurons. Action potentials were identified and analyzed using principal component analysis with custom Matlab software similar to previously published methods (Hengen *et al.*, 2009, 2011, 2012). To pharmacologically test for epsilon subunits, slices were sequentially exposed to 200 and 300 μM pentobarbital (45 min each). Following 300 μM pentobarbital application the $GABA_A$ agonist, muscimol (20 μM), was applied to confirm the presence of functional $GABA_A$Rs. Spontaneous firing rates were averaged into 5 min bins and normalized to a 30 min baseline period. Statistical differences were determined with a Kruskal-Wallis non-parametric one-way ANOVA test using normalized, average firing rates from the last 15 min of 300 μM pentobarbital application, $p<0.05$ was considered statistically significant.

IV. RESULTS

Daily MPA injections increased PBC-region neuronal resistance to pentobarbital.

To test whether MPA treatment (1.0 mg/day for 7 days; SQ) is sufficient to increase resistance to pentobarbital spontaneous firing rates were recorded from medullary slices taken from untreated (n=9 rats, 178 cells), vehicle control (n=6 rats, 87 cells), and MPA-treated (n=4 rats, 86 cells) rats. During 200 and 300 μM pentobarbital application normalized average firing rates were significantly higher at 79±10% of baseline in PBC-region neurons from MPA-treated rats compared to only 49-51% of baseline in untreated or vehicle control rats ($p<0.05$ for drug effect; $p<0.003$ compared to untreated and vehicle controls; Fig. 1A,B). In contrast, MPA treatment did not increase average firing rates in XII, NTS, or cortical neurons during pentobarbital application compared to untreated or vehicle controls ($p>0.05$; Fig. 1C-E).

To analyze whether the increased resistance to pentobarbital was due to increased firing rates all cells or a subpopulation, neurons were considered "resistant" if the firing rate was still >80% of baseline during the last 15 min of 300 μM pentobarbital. In PBC-region neurons the percentage of pentobarbital-resistant neurons increased to 45% from MPA-treated rats while only 13% and 16% of PBC-region neurons from untreated and vehicle control rats, respectively, were pentobarbital-resistant (Fig. 2A). In the XII motor nucleus, the percentage of resistant cells was to 28% of XII motoneurons in MPA-treated rats, 23% in untreated rats and 0% in vehicle controls (Fig. 2B). In the NTS, MPA treatment did not increase the percentage of pentobarbital resistant cells, as resistance decreased from 15% of NTS neurons in untreated rats to zero resistant cells in MPA-treated rats (Fig. 2C). Pentobarbital resistance in cortical neurons remained at only 3-7% across all rat groups (Fig. 2D).

Daily allopregnanolone injections increased PBC-region neuronal resistance to pentobarbital.

To test whether allopregnanolone treatment (1.0 mg/day for 2 days; SQ) is sufficient to increase resistance to pentobarbital, spontaneous firing rates were recorded from medullary slices taken from untreated and allopregnanolone-treated rats. During 200 and 300 μM pentobarbital application normalized average firing rates were significantly higher at 138±37% of baseline in PBC-region neurons from allopregnanolone-treated rats (n=2 rats, 17cells) compared to only 49 ± 5% of baseline in untreated control rats ($p<0.05$ for drug effect; $p<0.001$ compared to untreated controls; Fig. 3A, B). The percentage of pentobarbital-resistant PBC-region neurons increased to 44% of neurons from MPA-treated rats while only 13% of PBC-region neurons from untreated rats were pentobarbital-resistant (Fig. 3C).

V. DISCUSSION

This is the first study to suggest that pharmaceutically increasing central allopregnanolone levels is sufficient to increase epsilon subunit-containing $GABA_AR$s in PBC-region neurons. We found that daily MPA and allopregnanolone injections are sufficient to increase pentobarbital resistance in PBC-region neurons from only 13% of cells in untreated rats to ~45% of cells in MPA and allopregnanolone treated rats. Thus, increasing epsilon subunit expressing in $GABA_AR$ may be an important respiratory control mechanism to protect breathing from excessive inhibition when brain neurosteroid levels are increased. This is the first demonstration of the ability to pharmaceutically control epsilon subunit expression with neurosteroid administration. Thus, manipulating epsilon subunit expression may be a novel therapeutic target.

Epsilon subunits: expression and function

First cloned in 1997 (Davies *et al.*; Whiting *et al.*), the epsilon subunit confers many unique properties to $GABA_A$Rs such as increased resistance to positive allosteric modulators (Davies *et al.*, 1997), constitutive activity (Davies *et al.*, 1997), and altered desensitization properties (Wagner *et al.*, 2005). Epsilon mRNA expression is restricted to hypothalamus, hippocampus, medulla and spinal cord in monkeys (Whiting *et al.*, 1997), however, epsilon subunit mRNA is expressed throughout the rat brainstem, including the raphe nuclei, A5 area, NTS, locus coeruleus, XII motor nucleus, ventral respiratory column, PBC-region, and dorsal vagal complex (Moragues *et al.*, 2000; Kasparov *et al.*, 2001, Turner *et al.*, in preparation). Additionally, epsilon subunit mRNA increases in the medulla during development from embryonic day 14 to postnatal day 12 (Pape *et al.*, 2009). However, the role of epsilon subunit expression in native $GABA_A$Rs is not well understood.

Recently, our group has hypothesized that the epsilon subunit is increased during pregnancy in PBC-region neurons and XII motoneurons when brain allopregnanolone levels are increased 3-fold (Concas *et al.*, 1998, Hengen *et al.*, 2012; Chapter 2). In PBC-region neurons and XII motoneurons, resistance to pentobarbital increases during late pregnancy which suggests an increase in epsilon subunit expression. Further, when brain allopregnanolone concentrations are increased 3-4 fold from P10-P14 (Grobin and Morrow, 2001) pentobarbital sensitivity changes in a brain-region specific manner (Chapter 3). Thus, in native $GABA_A$Rs, epsilon subunit expression appears to increase in response to endogenous increases in central allopregnanolone levels. We hypothesize that increased epsilon subunit expression is a novel compensatory mechanism to protect respiratory-related neuronal function when GABAergic inhibitory signaling is enhanced. It is unknown whether epsilon subunit expression can be induced pharmaceutically to protect breathing from excessive neuronal inhibition.

Epsilon subunit incorporation in $GABA_AR$s can be increased pharmacologically

Is induction of epsilon subunit expression brain region specific? Here, we show that resistance to pentobarbital increases in PBC-region neurons following daily treatment with MPA or allopregnanolone. While MPA increases pentobarbital resistance in PBC-region neurons, pentobarbital resistance did not increase in NTS neurons, XII motoneurons or cortical neurons. Potentially, these data suggest there are differential thresholds for increasing epsilon subunit expression between various brain regions. Supportively, other preliminary data suggest epsilon subunit containing $GABA_AR$ increases in PBC-region neurons following 7 day isoflurane exposure, but, not in the cortex until after a 30 day exposure (Behan Lab, unpublished observations). Thus, increasing epsilon subunit expression in other brain regions may require different timing, duration, or dosage of drug administration.

Underlying mechanisms: MPA binds with high affinity to progesterone receptors and also increases brain allopregnanolone levels (Bernardi *et al.*, 2006). Therefore, it is unclear whether MPA increases pentobarbital resistance by acting on progesterone receptors or by increasing central allopregnanolone concentrations. Our preliminary data suggest that only 2 allopregnanolone injections (1.0 mg; SQ) are sufficient to increase resistance to pentobarbital in PBC-region neurons. While the mechanism of MPA-induced resistance remains unknown, taken together our data suggest that increasing brain allopregnanolone concentrations is sufficient to increase epsilon subunit expression in PBC-region neurons.

Physiological and clinical implications:

Increased brain allopregnanolone levels are hypothesized to be an endogenous neuroprotective mechanism against hypoxia (Billiards *et al.*, 2006), umbilical cord occlusion (Nguyen *et al.*, 2004),

intrauterine growth restriction (Westcott *et al.*, 2008), birth asphyxia (Fleiss *et al.*, 2012) and ischemia (Morali *et al.*, 2011). Furthermore, blockade of $GABA_A$Rs significantly attenuates the neuroprotective, allopregnanolone-dependent decrease in dopamine efflux during oxygen-glucose deprivation *in vitro* (Knight *et al.*, 2012). These data suggest that $GABA_A$R are an important target for eliciting neuroprotection. However, enhancing GABAergic inhibition via elevated allopregnanolone levels also poses the risk of excessively inhibiting neurons whose function is critical for life, such as inspiratory rhythm generating PBC neurons. Therefore, increased epsilon subunit incorporation in $GABA_A$Rs also appears to be an endogenous protective mechanism because it allows neuronal function to continue when allopregnanolone concentrations are increased. Future studies are needed to determine whether respiratory-related neurons increase epsilon subunit expression under other conditions that increase brain allopregnanolone concentrations such as ethanol (Follesa *et al.*, 2004) and inflammation (Billiards *et al.*, 2006). The ability to pharmaceutically control epsilon subunit expression in order to optimize neuronal excitability could have clinical implications for protecting breathing.

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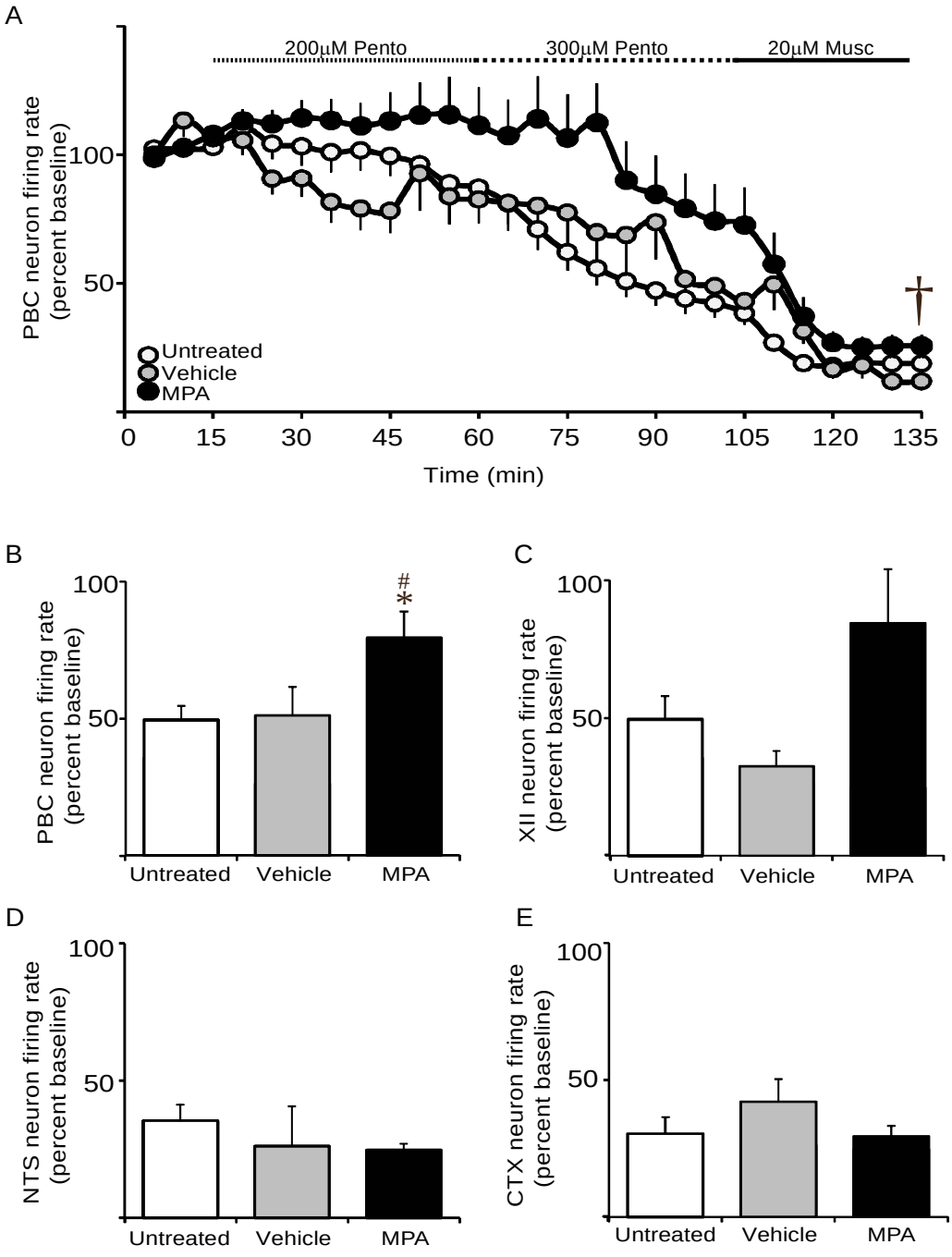
Figure 1: Daily MPA treatment increases pentobarbital resistance in PBC-region neurons. (A) MPA-treated rats (black circles) have significantly higher normalized average firing rates during pentobarbital application in PBC-region neurons compared to untreated (white circles) and vehicle controls (gray circles; $p<0.05$). (B)Average PBC-region neuron firing rate is significantly higher at 79±10% of baseline in neurons from MPA-treated rats (black bars) compared to only 49-51% in untreated (white bars) or vehicle control rats (gray bars). (C-E) XII (C), NTS (D), and CTX (E) neuron normalized average firing rates are not different between untreated (white bars), oil-treated vehicle control (gray bars) and MPA-treated (black bars) rats. * indicates $p<0.05$ compared to untreated controls; # indicates $p<0.05$ compared to vehicle controls † indicates drug effect $p<0.05$.

Fig. 1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200μM Pento
300μM Pento
20μM Musc
PBC neuron firing rate (percent baseline)
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50
Untreated
Vehicle
MPA
0
15
30
45
60
75
90
105
120
135
Time (min)
B
PBC neuron firing rate (percent baseline)
100
50
#
*
Untreated
Vehicle
MPA
C
XII neuron firing rate (percent baseline)
100
50
Untreated
Vehicle
MPA
D
NTS neuron firing rate (percent baseline)
100
50
Untreated
Vehicle
MPA
E
CTX neuron firing rate (percent baseline)
100
50
Untreated
Vehicle
MPA

Figure 2: Daily MPA treatment increases the percent pentobarbital-resistant PBC-region neurons.

(A) In PBC-region neurons from MPA-treated rats, the percentage of pentobarbital-resistant neurons increased to 45% while only 13% and 16% of PBC-region neurons from untreated and vehicle control rats, respectively, were pentobarbital resistant. (B-D) Neurons were categorized as resistant if firing rate was >80% of baseline during the last 15 min of 300 μM pentobarbital. In XII (B), NTS (C), and CTX (D) neurons the percentage of resistant cells remained relatively stable between untreated controls (white bars), oil-treated vehicle controls (gray bars), and MPA-treated (black bars) rats.

Fig. 2

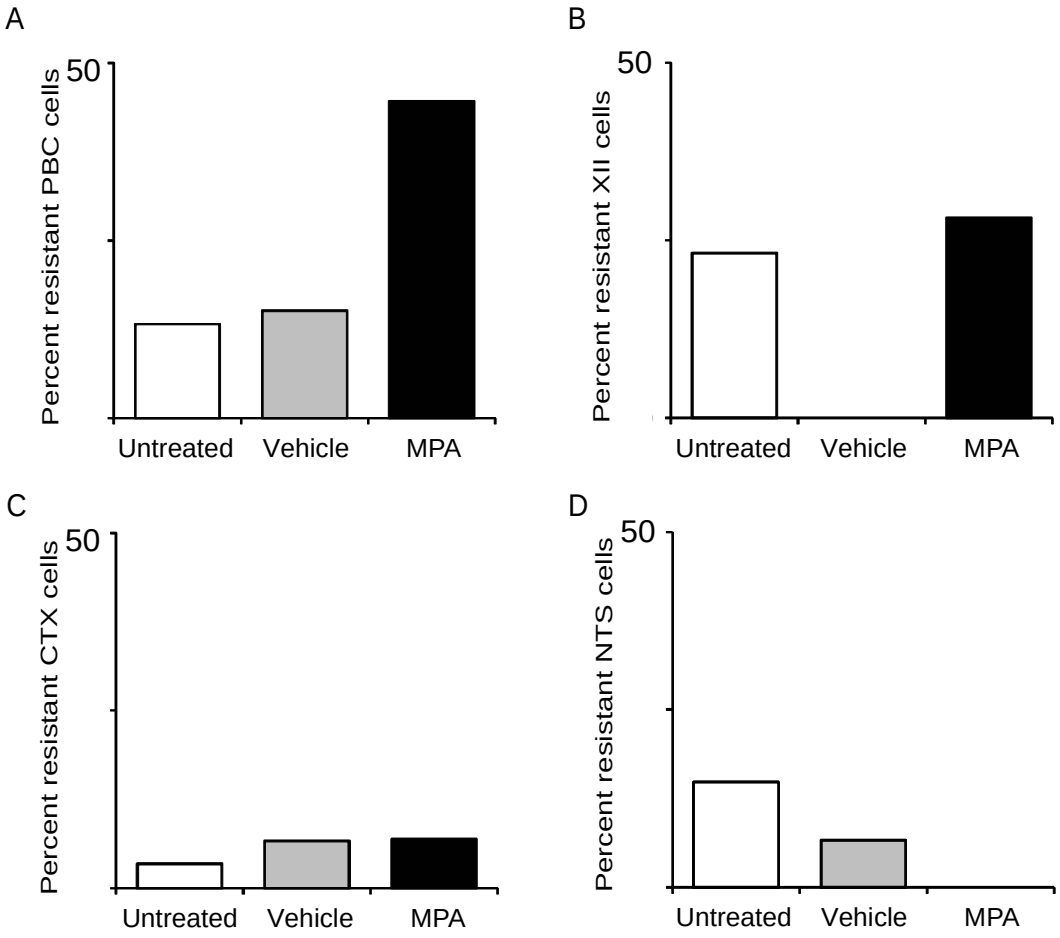
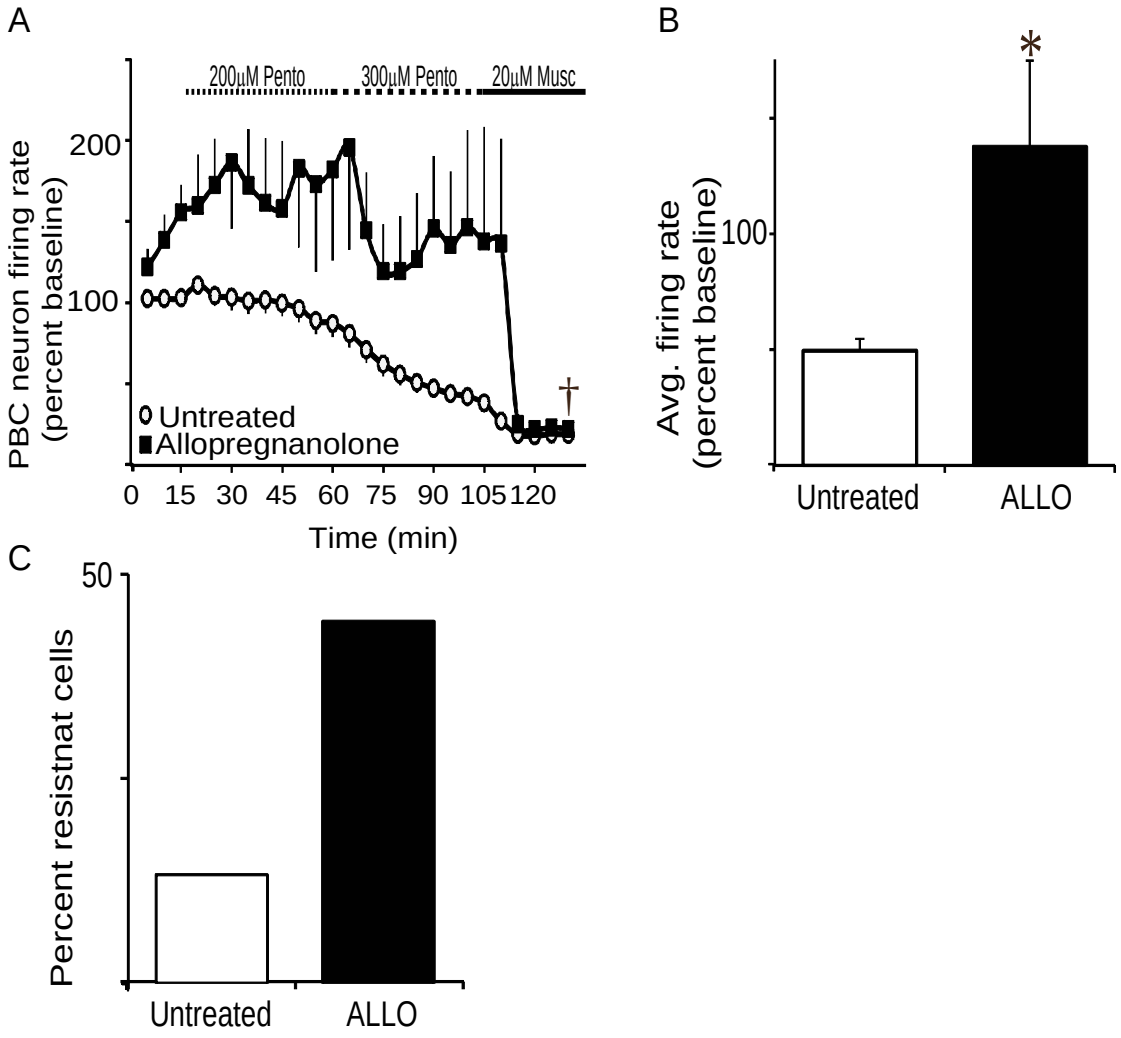

A
Percent resistant PBC cells
50
Untreated
Vehicle
MPA
B
Percent resistant XII cells
50
Untreated
Vehicle
MPA
C
Percent resistant CTX cells
50
Untreated
Vehicle
MPA
D
Percent resistant NTS cells
50
Untreated
Vehicle
MPA

Figure 3: Daily allopregnanolone injections increase PBC-region neuronal resistance to pentobarbital. (A) Allopregnanolone treatment (black squares) increases normalized average firing rates in PBC-region neurons during pentobarbital application compared to untreated rats (white circles; $p<0.05$). (B) PBC-region neuron normalized average firing rates from allopregnanolone-treated (black bar) rats were significantly higher at 138±37% of baseline compared to only 49±5% of baseline in PBC-region neurons from untreated control rats (white bar, $p<0.001$ compared to untreated controls), during the last 15 min of 300 μM pentobarbital application. (C) Neurons firing at >80% of baseline during the last 15 min of 300 μM pentobarbital were considered pentobarbital-resistant. The percent of resistant PBC-region neurons from allopregnanolone-treated (black bar) rats increased to 44% of neurons from only 13% in untreated rats (white bar). Symbols are as in Fig. 1.

Fig. 3


A
200μM Pento
300μM Pento
20μM Musc
PBC neuron firing rate (percent baseline)
200
100
Untreated
Allopregnanolone
†
0 15 30 45 60 75 90 105 120
Time (min)
B
*
Avg. firing rate (percent baseline)
100
Untreated
ALLO
C
50
Percent resistnat cells
Untreated
ALLO