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Role of Serotonin (5-HT) in Hypoxic and Hypercapnic Ventilatory Responses

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Abstract of the Dissertation

Role of Serotonin (5-HT) in Hypoxic and Hypercapnic Ventilatory Responses

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Breathing is fundamental to life, and responding appropriately to hypercapnic and hypoxic conditions is essential to survival. Abberant respiratory behavior is a feature of a wide variety of clinical conditions, and appears to be central and causative to the progression and outcome of some diseases, including Sudden Infant Death Syndrome (SIDS). Evidence of serotonergic (5-HT) abnormalities within the medulla are amongst the most robust and consistent pathological findings in SIDS victims. It is hypothesized that these underlying brainstem abnormalities precipitate the failure of normal, protective homeostatic responses to physiological stressors. Understanding the mechanisms by which 5-HT modulates the responses to hypoxia and hypercapnia, two potential stressors, is of paramount importance. It has been established that 5-HT exerts a strong, primarily excitatory, neuromodulatory effect on breathing, which may be accounted for by activation of the 5-HT_{2A} receptor. Recent studies have begun to

examine the role of 5-HT_{2A} receptor activation on eupnea and gasping; however, our understanding of this role is far from complete. Moreover, very little is known about the role of 5-HT_{2A} receptor-mediated modulation of the hypercapnic ventilatory response although considerable evidence supports a role for 5-HT neurons functioning as central CO_2 chemosensors. Furthermore, in our previous studies, we have found evidence to support an important role for gap junctions in CO_2 chemosensitivity, including the observation that mice deficient in gap junction protein connexin32 (Cx32) display an abnormal hypercapnic ventilatory response *in vivo*. While we have characterized and identified the presence of gap junction proteins in medullary raphe 5-HT neurons, their functional role is yet to be ascribed. This thesis research was designed to (1) evaluate the influence of 5-HT_{2A} receptor activation and blockade on phrenic nerve discharge under eupneic and hypoxic conditions using an arterially-perfused adult rat preparation, (2) examine the role of endogenous 5-HT_{2A} receptor activation in the hypercapnic ventilatory response using an arterially-perfused adult rat preparation, and (3) study the effects of chronic elevation of endogenous 5-HT on the hypercapnic ventilatory response in wildtype C57BL/6 and Cx32-deficient mice *in vivo*.

Table of contents

List of Figures

List of Tables

List of Abbreviations

5-HT- 5-hydroxytryptamine, serotonin

5-HT_{2A}-(R)- serotonin 2A (receptor)

ApEn- Approximate Entropy

BL- baseline

CON- control

Cx 32- connexin 32

DOI- 2,5-dimethoxy-4-iodoamphetamine

EMG- electromyogram

FLX- fluoxetine

HCVR- hypercapnic ventilatory response

HVR- hypoxic ventilatory response

SIDS- Sudden Infant Death Syndrome

T_I- Inspiratory burst duration

T_E- Expiratory duration

T_{PEAK}- Time to peak phrenic burst activity

VHCL- vehicle

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CHAPTER 1

INTRODUCTION

Role of Serotonin (5-HT) and Gap Junctions in Central Respiratory Control

SPECIFIC AIMS

This dissertation work is divided into two parts. The first part focuses on the role of serotonin (5-HT) neurotransmission in central respiratory control, and for this part, we hypothesized that 5-HT_{2A} receptor activation participates in the excitatory component of hypoxic and hypercapnic ventilatory responses. The second part focuses on the role of gap junctions in conjunction with serotonergic neurotransmission in central CO_2 chemoreception, and for this part, we hypothesized that gap junction coupling amongst 5-HT neurons plays an important role in modulation of the ventilatory response to increased levels of CO_2.

A significant body of research has established that 5-HT exerts a strong neuromodulatory effect on breathing, largely excitatory in nature. These effects can be accounted for by activation of the 5-HT_{2A} receptor. Recent studies have begun to examine the role of 5-HT_{2A} receptor activation on eupnea and gasping; however, our understanding of this role is far from complete. Moreover, very little is known about the role of 5-HT_{2A} receptor-mediated modulation of the

hypercapnic ventilatory response. Therefore, in Aim 1 we have conducted experiments to study the effects of a pharmacological 5-HT_{2A} receptor agonist and antagonist applied in the *in vitro* arterially-perfused adult rat preparation, during eupnea and hypoxia. While in Aim 2 we have conducted experiments in the same preparation to determine to role of endogenous 5-HT_{2A} receptor activation in the ventilatory response to hypercapnia.

Considerable evidence supports a role for 5-HT neurons functioning as central chemosensors. Our laboratory is interested in the many roles of gap junctions in neural control of breathing, including CO_2 chemoreception. In our previous studies, we have found evidence to support an important role for gap junctions in CO_2 chemosensitivity; notably, uncoupling of brainstem gap junctions *in vitro* abolishes the ventilatory response to increased levels of CO_2. Additionally, mice deficient in gap junction protein connexin32 (Cx32) display abnormal hypercapnic ventilatory responses *in vivo*. Furthermore, we have identified and characterized gap junction proteins in 5-HT neurons of the caudal medullary raphe. However, the role of these gap junctions, and whether they are involved in the release of 5-HT, and consequently the response to elevated CO_2, remained to be resolved. Therefore, in Aim 3, we conducted experiments in an *in vivo* mouse preparation to investigate the effects of chronically elevated levels of 5-HT on the hypercapnic ventilatory response in Cx32 knock-out mice.

AIM 1: *To evaluate the effects of 5-HT_{2A} receptor activation and blockade on eupnea and the hypoxic ventilatory response (HVR), including gasping.*

AIM 2: *To evaluate the effects of endogenous 5-HT_{2A} receptor activation on the ventilatory response to hypercapnia.*

AIM 3: *To identify the effects of chronically elevated serotonin (5-HT) on the ventilatory responses to hypercapnia, and to determine whether these responses are altered in a Cx32- deficient model.*

BACKGROUND AND SIGNIFICANCE

Neural Control of Breathing

Breathing is a fundamental process, essential to survival and homeostasis. Superficially, breathing appears to be relatively simplistic, often consisting of regular cycles of inspiration and expiration. However, the system is equipped to respond rapidly and sensitively to a variety of environmental alterations, both internal and external to the organism. While subject to voluntary control and manipulation, the initiation of breathing arises spontaneously from centers within in the brainstem, and this rhythm may be accelerated, slowed or otherwise altered, in response to systemic signals and inputs from higher brain centers.

The anatomy of the mammalian central respiratory controller is composed of a few key brainstem regions. In the medulla, two bilateral aggregations of respiratory neurons exist known as the dorsal respiratory group (DRG) and the

ventral respiratory group (VRG). These regions contain both inspiratory and expiratory neurons, making it difficult to define discrete inspiratory and expiratory centers (54). Extensive evidence exists to suggest that a subset of neurons located in the ventrolateral medulla in a region known as the pre-Botzinger complex (pre-BötC) display bursting pacemaker abilities, making them a candidate for the central rhythm generator of the respiratory system (43, 63, 153, 164, 167, 168). The regions of the parafacial respiratory nuclei or group (pFRG) and retrotrapezoidal nucleus (RTN), have also been suggested as a site for respiratory rhythm generation (13, 130-132). The mutual exclusivity or interdependence, as well as the relative developmental importance of these sites as the primary respiratory rhythm generator continue to be a topic for debate. It has also been suggested that the pFRG dominates expiratory rhythm while pre-BötC controls inspiration (84).

Nevertheless, an experimental preparation may be reduced, with regard to loss of upstream and downstream neural inputs, but so long as it captures the pre-BötC, it is still capable of generating various forms of inspiratory activity, including fictive eupnea, sighs, and gasps (115, 151, 164). Meanwhile, lesioning of pre-BötC neurons abolishes respiratory rhythm (63, 152, 204). Conversely stimulation of neurons within this region increases the frequency of inspiratory activity *in vivo*, and in some cases generates tonic excitation of inspiratory motor output (104, 108, 168, 171, 172), while blocking normal neurotransmission within the region also results in altered respiratory rhythm (21). Pre-BötC neurons are a

subset of a larger network of neurons in the brainstem, which contains both inspiratory and expiratory neurons, which govern breathing responses. These network neurons control the activity of spinal premotor and motoneurons via synaptic excitation and inhibition, ultimately innervating respiratory muscles and controlling breathing movements (17, 111, 154). Straddling the pre-BötC, in the most rostral region of the VRG, is the Bötzinger complex. These cells are responsible for reciprocal inhibition in the respiratory network, mainly utilizing GABA as an inhibitory neurotransmitter at its far reaching projections to premotor and motoneurons in other areas of the VRG and DRG, and to spinal motoneurons (54). This inhibition is proposed to be responsible for the periodic cessation of breathing efforts, and may be important to respiratory rhythm generation as a whole in conjunction with the pacemaking activities of cells from the pre-BötC(154).

The DRG itself, located in the nucleus tractus solitarius (NTS), appears to contain mostly inspiratory neurons which project primarily to the contralateral spinal cord. The glossopharyngeal and vagus nerves primarily project to the NTS, carrying afferent inputs relaying information both chemical (including arterial PO_2, PCO_2, and pH from carotid and aortic arterial chemoreceptors) and mechanical (such as pulmonary stretch receptors from the lungs and airways) (16, 115). Characteristics of breathing, including depth and frequency, can then be altered to maintain homeostatic acid/base and blood gas balance.

The respiratory cycle can be considered a type of oscillatory activity, that is often considered in three phases: inspiration (I), post-inspiration or early/passive expiration (E1), and late expiration (E2) (20). Therefore, the respiratory cycle may be further characterized by many of its temporal features, for example duration of inspiratory activity (T_I), duration of the expiratory period or time between inspiratory efforts (T_E), and respiratory frequency. During eupnea or normal basal breathing, the phrenic neurogram displays an inspiratory burst which typically manifests as an augmenting or ramp-like discharge pattern, peaking in late inspiration. Eupneic-like activity may be observed in both *in vitro* and *in vivo* preparations, and is thought to be the net product of cells displaying pre-inspiratory, post-inspiratory, or expiratory activities, as well as inspiratory cells with an augmenting depolarization pattern (99). These patterns are called fictive eupnea when displayed in reduced preparations, and are believed to result from an orderly recruitment of phrenic premotor and motor units throughout the duration of inspiration (2). They are characteristically produced by a normoxic or hyperoxic respiratory neural network.

In contrast, exposure to hypoxic or anoxic conditions results in a different characteristic pattern of respiratory activity that may include gasping. Gasping is defined by an abrupt onset, short duration, decrementing burst, where inspiratory activity is greatest within the first 50% of the inspiratory duration(177-179). For phrenic activity, gasps are typified by discharge of greater amplitude and shorter duration than that observed in eupnea, with decreased frequency of firing, due to

an increase in the duration between bursts (157). Gasps are one of the later features of the hypoxic ventilatory response (HVR). The HVR is characterized initially by an increase in breathing frequency (hyperpnea) and amplitude, a response mediated by hypoxic stimulation of the peripheral chemoreceptors, also referred to as the peripheral chemoreflex (PCR). This excitation is followed by respiratory depression which leads to a primary apnea, then gasping respiratory activity emerges and continues until terminal apnea or death. St.-John and Leiter (2003) found that in the *in vitro* arterially-perfused rodent preparation, features of gasping produced by hypoxia/ischemia are well conserved between this and *in vivo* preparations(175).

Central CO_2 Chemoreception

Normal eupneic breathing may be altered by changes in the extracellular environment, indicating that breathing is sensitive to input from chemoreceptor cells, which are activated by changes in pO_2, pCO_2, and pH. Hypoxia, or low pO_2, is sensed peripherally via the carotid body and transmitted through the carotid sinus nerve. While pCO_2 and pH sensors are also present in the carotid body, this information is primarily sensed through brainstem mechanisms. CO_2/pH are representative of the acid/base equilibrium of the blood, and must be maintained within a very narrow range by balancing breathing to metabolic activity (12, 55, 98, 115). Furthermore, feedback from chemoreceptors provides a tonic, chemical drive to breathe (32, 146, 183).

In spite of their critical and prominent role in altering and maintaining respiratory activity, the exact localization and identity of the central chemoreceptors has not been fully resolved. This is because while chemoreceptor sites contain chemosensitive neurons, they may be colocalized, or share functions with neurons involved in other aspects of respiratory control. Additionally, multiple widely distributed regions within the lower brainstem have demonstrated characteristics satisfying criteria of central chemoreceptor sites (12, 19, 31, 37, 98, 101, 116, 118, 173). Although CO_2 chemosensation was originally thought to originate from regions located at or near the surface of the ventral medulla (VMS; (101, 107, 160, 191), recent investigations suggest that CO_2 chemosensitivity is widely distributed at multiple brainstem sites. These sites include the NTS, locus coerelus, pre-BötC, fastigial nucleus, retrotrapezoidal nucles, and the midline medullary raphe (115). Recent evidence also suggests that additional CO_2 sensitive neurons may be present in another region proposed to function as an expiratory rhythm generator, the parafacial respiratory nucleus(133). As such, it has been difficult to link chemosensitive properties displayed by neurons *in vitro* to alterations in breathing *in vivo*. Furthermore, work by several investigators has indicated that the relative contributions of any individual site could be affected by experimental conditions including the intensity of stimuli used, as well as the state of arousal of the neurons themselves (60, 61, 117, 119-124, 126, 128).

In spite of their wide distribution, chemoreceptor sites display certain common key characteristics potentially identifying them as such. These neurons display

increased c-fos expression in response to elevated CO_2, and furthermore display increased firing rates in response to CO_2 *in vitro*. These findings must then be related to breathing *in vivo*, whereby focal acidification at these regions is demonstrated to stimulate breathing. It is important to note that each identified site on its own does not appear to fully account for the degree of sensitivity and magnitude of response to relatively small changes in stimulus seen in the intact organism. The multiplicity of sites speaks to the sophistication of the system and may allow for a greater degree of control during different metabolic and physical states of the organism, including sleep (115).

The exact stimulus which is sensed by these receptor cells appears to be both extracellular and intracellular pH; however which of these and to what extent, appears varied and site-dependent (14, 58, 59, 202, 203) Evaluating how this stimulus is sensed within the cell offers an additional slew of options, as there exists many candidate proteins which are sensitive to pH. These pH-sensitive proteins include: 1) inward rectifer K^+ channels (86), 2)ion transport proteins such as, the Na^+/H^+ exchanger subtype 3 (NHE3),(149, 205) , 3)TASK channels(14). and 4) low resistance gap junctions (40, 42, 170).

Gap Junctions, Respiratory Control, and Chemosensitivity

Gap Junctions are an important functional feature of many cells. Direct and indirect evidence exists to suggest that gap junctions may play an important

role in control of breathing via modulation of respiratory rhythmogenesis and inspiratory motoneuron synchronization (22, 51, 169). A noteworthy characteristic of chemoreceptor neurons, in both the dorsal brainstem and locus coereleus as well those in the carotid body, is that they are coupled by gap junctions. This property evidently bears consequences for central and peripheral chemosensation (1).

Gap junctions are channels that allow for direct cell-to-cell coupling; ions and small molecules can freely move from the cytoplasm of one cell to another. These channels are composed of connexons, one from each of the adjoining cells, which in turn are comprised of a hexameric assembly of structural proteins called connexins (Cx). Studies have revealed anatomical and electrical data supporting the existence of gap junctions in the solitary complex and locus coereleus (4, 12, 30, 41, 81, 82, 135, 190) The identity of the connexin proteins involved has also been elucidated. Solomon et al. found that in neurons of identified chemosensitive regions (including SC (NTS, dorsal motor nucleus of vagus), ventrolateral medulla (retrofacial nucleus, retrotrapezoid nucleus, ventral respiratory group, pre-BötC), caudal medullary raphe, and LC), the gap junction proteins Cx26, Cx36, and Cx32 were expressed (166, 174). As reductions in intracellular pH have been suggested as a cellular signal for hypercapnia, and gap junctions themselves confer either electrical and/or mechanical coupling properties to cells, these findings provide a potential explanation of how multiple chemosensitive neurons synchronize in response to elevations in CO_2.

Further evidence has suggested that gap junctions may also play an important role in neuronal -glial interactions (4). Extensive evidence exists to show that neuronal-glial interactions occur through the extracellular space. For example, the release of neurotransmitters alters glial membrane potentials and activate second-messenger cascades. (38, 48, 138, 196). The glia themselves have been demonstrated to both release and remove neurotransmitters, and maintain ionic balance in the extracellular space (7, 8, 18, 85). However, direct cell-cell coupling via gap junctions has been shown to result in electrical coupling between neurons and astrocytes (4, 62, 127). Furthermore, gap junctions between neurons and glia may also provide an important chemical conduit, allowing for passage of calicium, inositol triphosphate, or ATP (28, 29, 35, 36, 141, 142). These interactions may represent additional means of modulating neuronal activity, and possibly further facilitating neuronal synchronization. The relevance of neuronal-glial gap junction coupling to CO_2 chemosensation is being actively investigated.

While cell-cell coupling does not appear to be necessary for CO_2 chemosensitivity, in the single cell, integrated network, or intact animal (5, 53, 135) these functional studies have limited themselves to the regions of the NTS and LC. Unpublished data from our laboratory has suggested that aberrant hypercapnic responses exist in an anesthetized intact transgenic Cx32 deficient mouse. Additional unpublished data from the arterially-perfused adult rat has shown that perfusion with gap junction uncoupling agents, increases basal phrenic

burst frequency yet abolishes the response to elevations in CO_2. Further investigations are necessary to determine how gap junction coupling is contributing to function in the putative central chemoreceptors. We hypothesized in this dissertation that cell-cell coupling may interact, at least in part, at the level of serotonergic neurotransmission, playing an important role in synchronization of chemosensitive raphe neurons.

Neuromodulation and Serotonergic Neurotransmission

While the interplay between excitation and inhibition may be essential for respiratory rhythm generation, modulatory neurotransmission appears to confer the high degree of adaptability necessary for function of such a highly dynamic system. Neuromodulation has been defined as "the ability of neurons to alter their electrical properties in response to intracellular biochemical changes resulting from synaptic or hormonal stimulation(88). Respiratory neuronal networks are modulated by several neurotransmitters, including those that are considered classically excitatory (e.g., glutamate) or inhibitory (e.g., glycine), as well as through monoamine transmitters (*e.g.,* 5-HT).

5-HT is both a signaling molecule and a neurotransmitter, and in mammals, it is synthesized by enterochromaffin cells in the gastrointestinal tract and a select population of neurons in the CNS. These neurons are near and along the midline of the brainstem in the region known as the serotonergic raphe nuclei and typically produce Substance P and Thyrotropin Releasing Hormone alongside

5-HT (83). Neurons in all of the nuclei that govern respiratory control in the pons and medulla are innervated by 5-HT neurons(34, 77, 78, 180, 198). These projections originate from the medullary raphe nuclei and ventrolateral medulla (parapyramidal region) (34, 77, 102, 165, 188). Receptors for 5-HT are located both pre- and post-synaptically, and can also be localized extra-synaptically on the cell membranes of neurons and other cell types. These receptors hail from a broad range of the 5-HT receptor families: 5-HT_1–5-HT_7 receptors consist of multiple subtypes that differentially mediate either excitatory or inhibitory effects. All of the 5-HT receptors are G-protein coupled, seven transmembrane receptors, that activate an intracellular second messenger cascade, except for the 5-HT_3 receptor, which is a ligand-gated ion channel, and is therefore capable of fast synaptic transmission (79). The G-protein mediated changes in intracellular function alter the excitability of the effector cell, thus modulating the effects of any additional stimulus. Due to the multitude of receptor subtypes and wide variety of systemic effects amongst experimental models, the exact effects of 5-HT have remained unclear. 5-HT has been shown to produce a variety of responses, including facilitatory effects on the respiratory rhythm generator via medullary 5-HT_{1A} receptors, the recruitment of phrenic motoneurons via 5-HT_{2A} receptors, and inhibitory effects on the transmission of the central drive to phrenic motoneurons via presynaptic 5-HT_{1B} receptors(69, 100, 110). Therefore the role of 5-HT in respiratory control has been the source of much speculation over many years of research, having been at once deemed net inhibitory, stabilizing or

regulatory, and excitatory - the latter of the three appearing to have gained the most favor in recent years (158).

5-HT plays an important role developmentally in the maturation and regulation of neurons involved in respiratory control, even as early as the embryonic stage of development. Blocking medullary 5-HT_{1A} receptors in rats at embryonic day 18 (E18) induced respiratory arrest, while activating them increased breathing frequency, demonstrating a critical role for endogenous 5-HT (45). Transgenic mice have offered important insight into the absolute roles of 5-HT. Through use of a knockout mouse model lacking 5-HT transcription factor Pet-1 and a conditional knockout mouse model of the factor Lmx1b, investigators were able to generate mice lacking 70% and 99.0% of 5-HT neurons, respectively(47, 68, 159) . Neonatal Pet-1 KO mice display a slow and unstable respiratory rhythm. In adult males, the respiratory phenotype manifests as a blunted response to CO_2 (52, 68, 73, 74, 96), a feature also present in Lmx1b conditional knockout mice(74). These findings suggest that 5-HT neurons play an important role in establishing eupnea as well as the hypercapnic ventilatory response. Application of exogenous 5-HT rescues the response to CO_2 in Lmx1b deficient mice, suggesting that 5-HT may act in an additional capacity to enhance the hypercapnic chemosensitivty of non-5-HT neurons (74).

A subset of studies has focused on generating excess endogenous amounts of 5-HT, by preventing its uptake via the serotonin transporter (SERT) or its

degradation by monoamine oxidase A (MAOA). Mice lacking the SERT display an overall reduction in 5-HT neurons as a result of excess 5-HT, and they show a blunted response to CO_2 (97). In MAOA-deficient transgenic mice, endogenously elevated 5-HT disrupted the normal structure of the neurons in the phrenic motor nucleus, inducing marked structural changesthat included alterations in dendritic branching and spines (23). While 5-HT has been shown to exert regulatory trophic effects necessary for normal development of the neuronal network, Bou-Flores and colleagues further suggested that the structural abnormalities present in the transgenic mice reflected the internalization of 5-HT_{2A} receptors (5-HT_{2A}R) in response to their overactivation by excess 5-HT (23). The 5-HT_{2A}R is robustly expressed on phrenic motoneurons. When this receptor was pharmacologically blocked, phrenic motoneurons retained normal morphology.

The 5-HT_{2A}R subtype is commonly considered excitatory, although pharmacological perturbations at this receptor have yielded complex effects on respiratory activity(93, 109, 110). In the *in vitro* brainstem spinal cord preparation, application of specific 5-HT_{2A} agonists was demonstrated to exert an excitatory effect (134). These findings were replicated in more intact preparations including anesthetized cats (94) and conscious rats (26, 27).At the cellular level, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors typically couple with G_q proteins. These are coupled to phospholipase C (PLC), the activation of which generates inositol trisphosphate (IP3) and diacylglycerol (DAG). The end result is a release of Ca^{2+} from intracellular stores, which increases cytosolic Ca^{2+}, and the activation of

protein kinase C (PKC) (33, 39). Furthermore, activation of 5-HT_{2A}R results in elevations in persistent sodium currents, in addition to activating second messenger pathways (*i.e.* PKC) (144). The net effect of these intracellular changes is an increase in neuronal excitability.

As 5-HT itself has been shown to have facilitatory effects on respiratory activity, it has been postulated that 5-HT is tonically released by raphe neurons within the brainstem. This then acts on 5-HT_{2A} receptors within the many widely distributed raphe projection sites to support stimulation of respiratory activity. The 5-HT_{2A} receptor has been demonstrated to play a critical role in the establishment of a form of respiratory plasticity called long-term facilitation (LTF), whereby bouts of intermittent hypoxia lead to long-lasting increases in breathing well after the stimulus is removed (9, 10). In the neonatal mouse *in vitro* transverse medullary slice preparation, stimulating 5-HT release via AMPA-mediated activation of the raphe, bath application of 5-HT, and localized application of 5-HT to the pre-BötC have been shown to increase the frequency of hypoglossal or respiratory-related neuronal bursts(3, 148, 161). These effects can be mimicked by the application of the 5-HT_2 agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and blocked by the 5-HT_2 antagonist ketanserin (3, 144). Ketanserin not only blocks the 5-HT_{2A} receptor, but it also has affinity for the 5-HT_{2C} and α1 receptors. In the transverse medullary slice preparation, application of ketanserin has been shown to decrease both burst

frequency and amplitude, suggesting that 5-HT_{2A} receptors play an important role in the maintenance of respiratory activity within the pre-BötC (144, 148).

Conceivably, one of the major functions of neuromodulation is to alter the eupneic rhythm to meet physiological demands when the system is challenged. Evidence exists to suggest 5-HT modulates the respiratory response to hypoxia. 5-HT_{2A} receptor activation was demonstrated to be critical for generation of gasps in the neonatal mice in *in vitro* medullary slice preparations. Two types of pacemaker neurons are proposed to contribute to respiratory rhythm generation during eupnea, with one pacemaker reliant on a persistent sodium current for activation (cadmium-insensitive, or CI) and the other being sensitive to a calcium dependent current (Cadmium-sensitive or CS) (50, 187). Additionally, it appears that eupnea and gasping differentially rely on these different types of pacemaker neurons and 5-HT_{2A} receptor activation. Tryba and colleagues hypothesized that since CI pacemaker neurons require endogenous activation of 5-HT_{2A} receptors, 5-HT_{2A} receptor activation is critical for the generation of gasping (192). Application of 5-HT_{2A} receptor antagonists, piperidine or ketanserin, to the medullary slice preparation selectively eliminated gasping and CI pacemaker bursting. However, bursting of CS pacemaker neurons within the VRG was preserved with application of either agent (192). Additionally, during ketanserin-induced blockade of 5-HT_{2A} receptors, hypoxia in the arterially-perfused adult rat preparation produces fewer gasps and significantly delays

population bursting of neurons within of the VRG (176). This suggests that endogenous 5-HT_{2A} receptor activity may play an important role in restoring network rhythmicity and eupneic activity after hypoxic conditions.

5-HT_{2A} and CO_2

An additional stimulus that would require rapid changes in ventilation is a rise in arterial pCO_2, or exposure to hypercapnia. As peripheral chemoreceptors are located at sites that allow for rapid sampling of blood with gas content essentially identical to that from the heart (i.e., the carotid arteries and aortic arch), central chemoreceptors too must conceivably be located close to major blood vessels of the brain. 5-HT neurons of the midline medullary raphe and in the rostral and caudal chemosensitive zones of the VLM are closely associated with the basilar artery and its largest branches (24).

5-HT neurons also display intrinsic chemosensitive properties. Typically, neurons from brain regions not involved in respiratory control are either unaffected or inhibited by acidosis (199, 200). Yet 5-HT neurons increase their firing rate in response to increasing CO_2 in both brain slices that have been synaptically isolated (via blockade of fast glutamate and GABA receptors) and in cultured raphé neurons ((199, 201) from the rat and mouse. Furthermore, 5-HT neurons physically isolated via dissection also display chemosensitive properties (162). This strongly suggests that chemosensitivity of medullary 5-HT neurons is not the result of upstream input from a different brainstem region.

Stimulation 5-HT neurons by CO_2 has also been documented *in vivo*, through chronic neural recordings (194, 195) as well as demonstration of increased c-fos expression in medullary neurons that also display the serotonergic marker tryptophan hydroxylase (TpOH) (66, 67, 87, 95, 129, 145, 186) upon exposure to hypercapnia. Furthermore, focal acidification of the medullary raphe increases ventilation *in vivo* in several animal models(19, 71, 72, 120). A similar approach using in vitro medullary slices shows increased hypoglossal motor output in response to acidification of medullary raphe cells (140, 148). Moreover, the importance of midline 5-HT neurons in central chemosensation is highlighted by evidence indicating that the inhibition or destruction of these neurons leads to a loss or blunting of the hypercapnic ventilatory response(46, 71, 105, 106, 113, 125, 185).

Clinical Significance

Breathing is fundamental to life, and responding appropriately to hypercapnic and hypoxic conditions is essential to survival. Deranged respiratory behavior is a feature of a wide variety of clinical conditions. In some of these, it appears that the pathology of breathing may actually be central and causative to the progression and outcome of disease. Sudden Infant Death Syndrome, is one such example, wherein apparently healthy infants under one year of age inexplicably die, typically while in sleep (91). Currently, SIDS is third leading cause of infant death between birth and 12 months of age, after congenital

malformations and low birth weight. In the United States, however, SIDS is the number one killer of infants in the same age group (80, 103). These figures reflect a reduction in the incidence of SIDS ~50%, which resulted from the observations that placing an infant to sleep in the supine, rather than prone, position could decrease the risk of SIDS (206). Clearly, SIDS still poses a major threat to infant survival, particularly amongst racial and ethnic demographics suffering from poor access to healthcare and health education (90). The necessity to understand more about the physiological etiology of SIDS has never been more apparent, and several promising leads exist. One important hypothesis has focused on the underlying brainstem abnormalities which potentially precipitate a failure of normal protective homeostatic responses to physiological stressors (90). A major component of this hypothesis involves 5-HT, as evidence of 5-HT abnormalities within the medulla are amongst the most strong and consistent findings in current SIDS brainstem research (89, 136, 137, 139). While great scientific strides have been made in understanding many aspects of 5-HT development, and its homeostatic significance, more work remains to be done. Of relevance to this dissertation, additional information is needed to understand the mechanisms by which 5-HT modulates the response to hypoxia and hypercapnia, two potential stressors that may play a role in pathogenesis of SIDS.

In spite of a significant body of evidence identifying respiratory related regions of the medulla and spinal cord, including distinct neuronal populations, that are responsive to alterations in 5-HT levels by 5-HT_{2A} receptor activation,

many aspects of how 5-HT_{2A} receptor activation may modulate the generation of respiratory rhythm remain unresolved, especially under conditions of severe hypoxia and hypercapnia. In spite of mounting evidence in support of 5-HT neurons of the medullary raphe functioning as chemosensors, to date it is unknown if the hypercapnic ventilatory response is hinged upon 5-HT_{2A}R activation. While the apparent inconsistencies between studies may be attributed to a number of causes including differences in experimental technique, model species, and the maturity and relative intactness of neural substrate, thus far the neuronal network in which these receptors operate, specifically the role of cell-cell coupling in serotonergic modulation of respiratory activity, has remained largely ignored (See Fig.I.1). Gaining this important insight will increase our ability to understand the conundrum of how abnormalities within the serotonergic system may lead to catastrophic consequences in early life (i.e. SIDS). Therefore, this dissertation includes experiments aimed at understanding the involvement of 5-HT in eupnea and responses to hypercapnia and hypoxia. Specifically, we seek to establish the effect of endogenous and exogenous 5-HT_{2A} receptor activation on these respiratory behaviors, and to determine if endogenously elevated 5-HT can enhance the response to hypercapnia, particularly in cases where there is loss of Cx-32 mediated cell-cell coupling.

Figure I.1

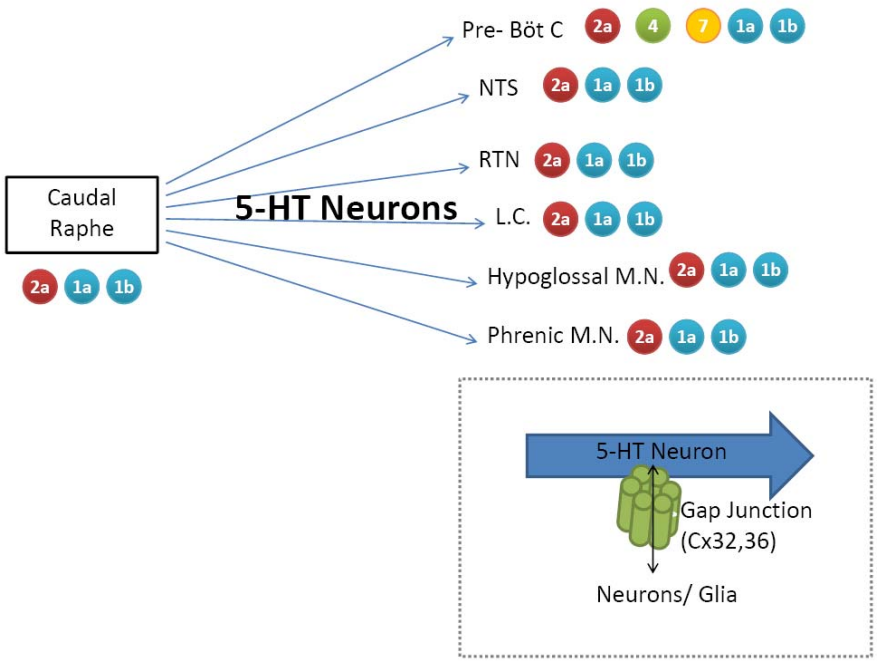


Figure I.1. Schematic of anatomic characteristics of the 5-HT respiratory network. 5-HT neurons of the caudal medullary raphe project to all major respiratory nuclei, including those depicted here. These nuclei have been demonstrated to express 5-HT receptor subtypes as shown (colored circles) though this information is incomplete. Note: reciprocal projections from the nuclei to the raphe and amongst the respiratory nuclei also exist but are omitted here for clarity. Inset: 5-HT neurons within the raphe express gap junction proteins connexin 32 and 36, allowing for cell-cell coupling between these neurons and neighboring cells.

CHAPTER II

GENERAL METHODS

Specific Aims 1 and 2

***Surgical Preparation*:**

For these experiments, Sprague-Dawley rats (75-140 g, n=78) were deeply anesthetized with 2-5% isoflurane and sub-diaphragmatically transected. The rostral tissue portion was placed in an ice-cooled bath containing artificial cerebrospinal fluid (aCSF; see below for composition) bubbled with 95% O_2/5% CO_2. The calvarium of the skull was then removed to expose the brain, for subsequent decerebration at the pre-collicular level. The lungs were removed and the descending thoracic aorta dissected from the ventral surface of the vertebral column, after which the tissue were transferred to a recording chamber. The descending aorta was cannulated with a double lumen catheter (French 3.5), and retrogradely perfused with aCSF containing an oncotic agent (Ficoll 70; 2.5%; Sigma Chemical Co.). Perfusion pressure was be generated by a peristaltic pump (Peri-Star; World Precision Instruments). One lumen of the catheter was used to perfuse the preparation and while the other was used to monitor perfusion pressure.

The aCSF was composed of (in mM): 1.25 $MgSO_4$, 1.25 KH_2PO_4, 5.0 KCl (unless otherwise specified), 25 $NaHCO_3$, 125 NaCl, 2.5 $CaCl_2$, and 10 D-

glucose. This perfusate was continuously gassed, warmed to 31°C, passed through a bubble trap (to remove bubbles and dampen pulsations from the pump), and filtered using a nylon mesh (pore size: 40 μm; Millipore) before entering the descending aorta. Perfusate draining from the preparation, was collected into the original reservoir, reoxygenated, and recirculated. The revolution rate of the pump was gradually increased to re-establish cardiac activity, followed by the onset of rhythmic contraction of the respiratory muscles. To eliminate respiratory-related muscle movements, a paralytic agent (vecuronium bromide; 1 mg), was added to the perfusate. In each experiment, one or both phrenic nerves was isolated, dissected, and transected at its insertion point on the diaphragm. The cut nerve(s) were then placed over a bipolar platinum rod hook electrode, and covered with a mixture of mineral oil and petroleum jelly to prevent drying. Flow was increased further at a slow rate until phrenic nerve activity displayed a eupneic (*i.e.*, ramp-like) discharge pattern. Phrenic nerve activity, temperature, and perfusion pressure were given adequate time to stabilize before beginning the experimental protocol,.

Data Acquisition and Analysis:

Phrenic nerve output signals were amplified (10k), notch filtered at 60 Hz, and analog filtered between 10 Hz and 1 kHz. Filtered signals were rectified, and a moving average obtained using a third-order Paynter filter with a 50-ms time constant. The raw and moving-averaged phrenic nerve signals were recorded on a computer at a sampling rate of 2 kHz (Chart 5.0, PowerLab, ADInstruments) and

on digital tape at a sampling rate of 2.5 kHz (DAT, CygnusTechnologies) for off-line analyses (MatLab 7.0.1).

Temporal Analyses:

Individual traces of time series data from the PowerLab recordings were output for temporal analyses. Inspiratory burst duration (T_I), the duration between bursts (T_E), burst frequency, burst amplitude, and the time-to-peak (T_{peak}) were determined for basal phrenic nerve discharge (baseline and recovery) and phrenic nerve activity during hypercapnia. Burst amplitude data was normalized to baseline levels of discharge, which was set to 100% in each preparation, and T_{peak} was normalized to T_I (T_{peak}/T_I, expressed as a percentage). Average values for these variables were calculated from 10 consecutive respiratory cycles during the last minute of each condition. For the anoxic trials, the duration of the PCR, apnea, and gasping was also determined. The onset latency to gasping, the number of gasps elicited for a constant duration of ischemia, and the total number of gasps elicited was evaluated.

Approximate Entropy Analyses: The complexity of a signal can be quantified using *approximate entropy* (ApEn) analysis, which provides a statistical index that measures and quantifies the regularity (orderliness) in a time series. Higher values of ApEn are associated with irregularity and greater randomness, and thus reflect less system order or higher system complexity. Conversely, lower values of ApEn are associated with a higher degree of regularity and predictability, and

thus reflect an ordered system or lower system complexity. ApEn was calculated as a function of the parameters *m,* the embedding dimension, and *r*, the tolerance (threshold) level. For the arterially perfused adult rat, *m* and *r* were set to the following values: arterially-perfused adult rat preparation 3 and 0.3 xSD, respectively. ApEn was calculated according to the methods described by Pincus (1991).

Statistical Analyses:

All data is reported as mean±SE. For data that are presented as paired data (*i.e.*, before and after design), statistical significance was assessed using one- and two-way analysis of variance (ANOVA) with repeated measures and the nonparametric Friedman's test, followed by *Holm-Sidak post hoc* analyses for multiple comparisons. For data that are presented in an unpaired design, statistical significance was evaluated using one- and two-way ANOVA and the nonparametric Friedman's test, followed by *Holm-Sidak post hoc* analyses for multiple comparisons. The criterion level for determination of statistical significance will be set at $P<0.05$.

CHAPTER III

SPECIFIC AIM I

The effects of 5-HT_{2A} receptor activation and blockade on eupnea and the hypoxic ventilatory response (HVR), including gasping.

Introduction

Neuromodulatory peptides, particularly serotonin (5-HT), have been suggested to play an important role in numerous respiratory-related behaviors. 5-HT activates multiple receptor subtypes, and thus, is capable of eliciting both excitatory and inhibitory influences on respiratory output. It appears that one important function of 5-HT neuromodulation is to provide a tonic excitatory influence on the respiratory network. Previous studies suggest that 5-HT acting on the 5-HT_{2A} receptor exerts an excitatory effect on the respiratory neural control system; however, recent studies have demonstrated that both activation and blockade of these receptors may elicit similar effects on eupnea, and that the role of 5-HT_{2A} in eupnea is not identical in all preparations studied thus far. The precise role of 5-HT_{2A} receptor activation and blockade in hypoxic/ischemic responses remains similarly undetermined.

To better understand the excitatory functions of 5-HT, studies have examined the role of activation of 5-HT_{2A} receptor subtype using 5-HT_{2A} receptor selective agonists; however, the responses are somewhat variable. In neonatal and

adult rats *in vivo*, intraperitoneal administration of the 5-HT_{2A} receptor selective agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) decreased tidal volume in both age groups but decreased respiratory frequency in neonates while increasing respiratory frequency in adults (27). Additionally, in the *in vitro* rat brainstem-spinal cord preparation, bath application of 5-HT or DOI increases the frequency of both hypoglossal nerve and cervical root (phrenic) discharge while decreasing burst amplitude (112). In the *in vitro* mouse transverse medullary slice preparation, bath application of DOI similarly increases inspiratory burst frequency; however, in this preparation, an increase in burst amplitude and an increase in the incidence of augmented (sigh) bursts were also noted (144). Although the above studies demonstrate excitatory effects on the timing of respiratory motor output, the influence on inspiratory burst complexity remain unknown.

On the other hand, the effects of receptor blockade are inconsistent between preparations. In the neonatal mouse transverse medullary slice preparation, for example, bath application of the 5-HT_{2A} receptor antagonist ketanserin (KTN) depresses or abolishes respiratory bursts (144). While in the arterially-perfused juvenile/adult rat preparation, blockade of 5-HT_{2A} receptors increases basal respiratory burst frequency (176).

Gasping in response to severe hypoxia/ischemia appears to be an essential mechanism for survival. The role of 5-HT_{2A} receptor activation in the genesis of

hypoxic/ischemic gasping has been explored in a number of *in vitro* and *in vivo* preparations; again, the observations are somewhat varied. Previous studies have suggested that serotonergic neurotransmission participates in the establishment and maintenance of gasping; Yet, the influence of exogenous activation of 5-HT_{2A} receptors on gasping has not been explored. Meanwhile, in the mouse slice preparation, 5-HT_{2A} receptor blockade abolishes anoxic gasping (192) while during blockade in the arterially-perfused rat, ischemic gasps persist, though modified (176, 189)

Together, these observations indicate that endogenous 5-HT acting on 5-HT_{2A} receptors may play an important role in eupneic and gasping respiratory activities, yet this role is not identical among different *in vitro* rodent preparations. The current study aimed to investigate the role of 5-HT_{2A} receptors in the ischemia-induced HVR and gasping as well as to re-evaluate their role in eupnea. Therefore, we evaluated the effects of 5-HT_{2A} receptor activation and blockade by DOI and KTN, respectively, on the temporal charactristics of phrenic nerve discharge and inspiratory neural network complexity during eupnea and ischemia-induced gasping. For these experiments, we selected the arterially-perfused adult rat preparation as the experimental model because it is more mature than other reduced preparations and contains an intact respiratory neuroaxis. We hypothesized that activation of 5-HT_{2A} receptors would enhance respiratory-related activity; this would be reflected as an increase in gasp number, with both eupneic bursts and gasps displaying an increase in frequency and

amplitude, as well as a reduction in inspiratory neural network complexity, concurrent with increased synchronization. Meanwhile, depression of respiratory-related activity and alterations in gasping would accompany the loss of endogenous 5-HT_{2A} activation under KTN.

Methods:

To evaluate the role of the 5-HT_{2A} receptor in eupnea, the hypoxic ventilatory response (HVR) and gasping, the arterially-perfused adult rat preparation was used (n=31). The methods and procedures for this experimental preparation have previously been discussed in detail, and will not be repeated here (see General Methods).

Two series of experiments were performed. In the first series, the effects of 5-HT_{2A} receptor activation using DOI on eupnea and the hypoxic ventilator response (HVR) was examined (n=11). In the second series, we evaluated the effects of 5-HT_{2A} receptor blockade using KTN on eupnea and the HVR (n=8). .Note that the 5-HT_{2A} receptor agonist DOI has also been shown to activate the 5-HT_{2C} receptor. However, this DOI-induced activation of 5-HT_{2A} and 5-HT_{2C} receptors has been suggested to be dose-dependent, with 5-HT_{2C} receptors being activated at higher doses, and yielding opposing effects in some neural systems (65). The 5-HT_{2A} receptor antagonist KTN has also been shown to block the 5-HT_{2C} receptor and the α_1-adrenoceptor (114).

Preliminary experiments have been conducted to identify an optimal drug concentration and time point for the effects of DOI. In these experiments, the preparation was continuously perfused for 10 min with normal aCSF followed by perfusion with aSCF containing DOI at either 833 nM, 1.67 μM, 3.33 μM, 6.66 μM, and 16.65 μM final concentration for 35 min (data not shown). From these experiments, a dose of 3.33 μM final concentration was selected, and with this dose, steady-state effects were noted by 10 minutes of perfusion; thus, for our subsequent experiments, the 3.33 μM concentration and perfusion time of 10-min were used. Selection of the dose and time point for effects of the antagonist KTN were selected based on previous dose-response studies conducted by our laboratory, which established that 40 μM final concentration of KTN is appropriate for this preparation.

In both series of experiments, phrenic nerve discharge was recorded under baseline conditions and during an ischemic challenge in both the presence and absence of the 5-HT_{2A} receptor agonist or antagonist. Our recording protocol consisted of recording stable baseline phrenic nerve discharge for at least 10-min, after which the preparation was perfused with DOI or KTN for 10 minutes. The bypass was then opened for ~90 s to produce ischemia.

Control experiments:

Since both DOI and KTN exert long-lasting effects, an additional series time control experiments was also conducted (n=12). In this case, basal phrenic

nerve discharge was recorded for 20 min during perfusion with normal aCSF prior to the ischemic trial. Due to the marked changes in gasping and HVR noted in preliminary experiments, we evaluated the effects of KTN on gasping using a paired paradigm, wherein at least 10 minutes of stable baseline was recorded, before opening of the bypass to induce ischemia for ~90s. At this point the bypass was closed and the preparation reperfused for at least 10 minutes until a stable, consistent baseline reappeared, before the addition of KTN to the perfusate. The recording protocol was repeated under KTN, thereby allowing each experiment to serve as its own control.

The experiments evaluating the effects of KTN on eupnea and HVR including gasping were repeated using aCSF with different extracellular K^+, than is typical for this preparation (6.25 mM). Experiments were conducted using ACSF with extracellular K+ concentration of 4.25 (n=8) and 3 mM (n=8). This data, along with the KTN data presented in this chapter (using 6.25 mM K^+), was previously published (11)and appears in the appendix of this document.

(See ***General Methods*** for *Data Acquisition and Analysis*)

Temporal Analyses:

Individual traces of time series data from the PowerLab recordings were output for temporal analyses. Inspiratory burst duration (T_I), the duration between bursts (T_E), burst frequency, burst amplitude, and the time-to-peak (T_{peak}) were

determined for basal phrenic nerve discharge (baseline and recovery). Burst amplitude data was normalized to baseline levels of discharge, which was set to 100% in each preparation, and T_{peak} was normalized to T_I (T_{peak}/T_I, expressed as a percentage). Average values for these variables were calculated from 10 consecutive respiratory cycles during the last minute of each condition. For the anoxic trials, the duration of the PCR, apnea, and gasping was also determined. The onset latency to gasping, the number of gasps elicited for a constant duration of ischemia, and the total number of gasps elicited was evaluated. As the number of gasps varied, gasp characteristics were evaluated over a 10 second interval.

Approximate Entropy Analyses:

The complexity of the phrenic nerve discharge was quantified using *approximate entropy* (ApEn) analysis (see General Methods). For an individual experiment, ApEn was calculated for each burst within 10 consecutive respiratory cycles, during the last minute of each condition, and averaged over the 10 bursts for each condition.

Statistical Analyses: (See *General Methods*)

Results

Temporal effects of DOI and KTN during eupnea

Both 5-$HT_{2A/2C}$ receptor activation by DOI (n=11), and blockade by KTN (n=8), resulted in marked changes in basal phrenic nerve discharge (Figure

III.1A). Interestingly, both perturbations resulted in an increase in phrenic burst frequency (~127% increase under DOI, P<0.001: ~43% increase under KTN, P=0.001). However, the elevation in frequency is engendered differently with each drug. Under DOI, T_E is reduced exclusively (P<0.001) and burst duration remains relatively unchanged (P=0.067), while under KTN both T_I and T_E are markedly reduced (T_I from 805.2±59.0ms to 497.3±13.8ms, P<0.001: T_E from 3.62±0.42s to 2.66±0.18s, P<0.001, Figure III.1B). No changes in temporal characteristics occurred during the course of the time control experiments.

Effects of KTN and DOI on phrenic burst characteristics and complexity

5-$HT_{2A/2C}$ receptor activation and blockade exert differential effects on characteristics of the phrenic bursts themselves. Both perturbations do result in marked reductions in amplitude (amplitude of phrenic bursts under DOI are ~40% of baseline amplitude, ~56% under KTN, Figure III.2.B). Furthermore under both conditions burst shape is changed by a shift in peak activity from the lattermost portion of the burst (as seen in the augmenting or ramp-like shape of eupneic discharge) to an earlier timepoint. However, the influence of KTN results in bursts characterized by a distinct bell-like shape (Figure III.2.A/B). Meanwhile, amplitude and burst shape were maintained throughout the course of time control experiments.

Approximate entropy (ApEn) is considered a measure of disorder within a system, whereby smaller values represent a greater degree of "similarity" within

the data and therefore, greater order or synchronization (163). Both over time, and under the influence of DOI, no detectable differences in ApEn occur. However perfusion with KTN results in a striking fall in ApEn (~12% reduction from baseline, P=0.015, Figure 2.C).

Effects of DOI on the Hypoxic Ventilatory Response (HVR) including Gasping

In this preparation gasping is elicited by the opening of a bypass, redirecting perfusate away from the preparation, causing it to become hypoxic/ischemic. The HVR is characterized by several distinct phases: (1) respiratory excitation (peripheral chemoreflex, PCR) with enhanced phrenic burst amplitude and frequency, and continued augmenting burst discharge pattern. (2) a period of transition bursts and/or apnea, and (3) gasping, characterized by decrementing discharge pattern and markedly prolonged duration between bursts(Figure III.3). Interestingly, in spite of the marked changes during eupnea under perfusion with DOI, the phases of the HVR and gasping appear to remain intact (Figure III.3).

Characteristic of the gasps themselves, including temporal characteristics (frequency, T_I, and T_E), burst shape (T_{PEAK}/ T_I), and complexity (ApEn) are conserved between DOI and control conditions. As gasp amplitude is typically considered as normalized to the pre-ischemic baseline levels, DOI-influenced gasps are significantly elevated (115.5±9.8% of baseline amplitude under control

vs. 373.9±71.7%, P=0.001). When normalized to pre-DOI basal amplitude, no significant difference emerges (not shown, P=0.347), suggesting that the apparent enhanced amplitude of DOI-influenced gasps is largely generated by the reduced amplitude of eupneic bursts under DOI, and not an increase in DOI-influenced gasp amplitude itself (Figure III.4.A).

The HVR can also be analyzed for the duration and timing of each of the phases of the response. Duration of the PCR, time to the onset of gasping (from both the opening of the bypass and the end of the PCR), duration of gasping, and the total number of gasps elicited are not significantly different between control conditions and under perfusion with DOI (Figure III.4.B)

Effects of KTN on the Hypoxic Ventilatory Response (HVR) including Gasping

In contrast to the minimal effects of DOI on gasping and the HVR, perfusion with KTN resulted in significant changes. In these experiments, under control conditions, the HVR was characterized by three phases: (1) respiratory excitation (peripheral chemoreflex, PCR) dislaying the typical elevation in phrenic burst amplitude and frequency, and continued augmenting pattern of burst discharge (2) a transitional period composed of transition bursts and/or apnea, and (3) gasping, where gasps are characterized by decrementing discharge pattern and markedly prolonged duration between bursts (Fig. III.5.A).

In all experiments, perfusion with KTN disrupted the typical phases of the HVR; the PCR was followed immediately by a series of ischemic bursts exhibiting a decrementing discharge pattern, but were not accompanied by a marked prolongation of T_E, with the abolishment of the transition/apneic period (Figure III.5.A). Note, that these changes were not influenced by the level of $[K^+]_o$ (Appendix). Our data demonstrate that with KTN, ischemia induces phrenic bursts that share burst timing and patterning characteristics with those of hypoxia/ischemia-induced gasps (decrementing burst pattern, Fig III.5.B), yet are not separated by long expiratory pauses (T_E). Under both control condition and perfusion with KTN, a similar number of phrenic bursts were elicited during the ischemic challenge (from the onset of the PCR), although under KTN these bursts are occurring over shortened timeframe. It is unclear if these burst may be identified as gasps, *per se*.

Discussion

The present results indicate that both endogenous and exogenous activation of 5-$HT_{2A/2C}$ receptors alters the general timing and patterning characteristics of basal phrenic nerve discharge. Consistent with previous findings, DOI exerts a generally stimulatory effect on breathing, manifested by an increase in phrenic burst frequency. This provides further confirmation that the excitatory effects of 5-HT on breathing may be accounted for at least in part by 5-HT_{2A} receptor activation.

Further, responses dependent on respiratory excitation, such as hypoxic and hypercapnic ventilator responses, may be dependent on some degree 5-HT_{2A} receptor activation. However, our data have shown that exogenous excess of 5-HT_{2A} activation by DOI does not significantly alter the timing of the hypoxic ventialtory response or characteristics of gasping. If the 5-HT_{2A} R is involved in establishment of the HVR and gasping, the degree of activation necessary must be maximally supplied by the ischemic/hypoxic challenge under control conditions.

The experiments involving KTN suggest that endogenous activation of 5-HT_{2A} receptors participates in establishing the general timing and patterning characteristics of basal phrenic nerve discharge. Interestingly, blockade of 5-$HT_{2A/2C}$ produced alterations during eupnea which shared many of the characteristics produced by activation by DOI. In contrast to findings from mouse medullary slice, KTN produced an increase in phrenic burst frequency and reduction in burst amplitude. Incidentally, these observations are consistent with previous reports from the arterially-perfused juvenile rat. To our knowledge we are the first to replicate any findings from neonatal mouse medullary slice in arterially-perfused adult rat. For example, the reduction in burst duration (T_I) during eupnea, was also reported with KTN in previous studies from neonatal mouse medullary slice (150), although burst frequency is reduced in these preparations.

That blockade of 5-$HT_{2A/2C}$ receptors via ketanserin resulted in an increase in frequency (excitation) is in contrast to findings from the mouse medullary slice, as well as counterintuitive in light of the respiratory excitation produced by 5-$HT_{2A/2C}$ receptor activation with DOI. A number of different possibilities exist as to why 5-$HT_{2A/2C}$ receptor blockade might produce respiratory activation. KTN could potentially block the activity of 5-HT neurons projecting to inhibitory neurons (i.e. GABAergic) to produce loss of inhibition and thereby unmasking respiratory excitation. The neuromodulatory nature of 5-HT suggests that some degree of 5-HT neuron activation might be present throughout eupnea. Here, we have only blocked 5-$HT_{2A/2C}$ receptors and therefore, it is also possible that the excitation is being produced by persistent activity of additional excitatory molecules co-released with 5-HT (i.e. Substance P and TRH). Finally, our observations may be the result of reciprocal function of additional neurotransmitter systems that might produce effects that are stimulatory to breathing, such as norepinephrine.

Over the years, *in vitro* preparations have been used to study the roles of various 5-HT receptor subtypes in respirator related behavior, with inconsistent results. Several differences between these studies and preparations exist, including species (mouse vs. rat) and age (neonatal vs. adult) of the preparation, amount of neural substrate (slice vs. relatively intact neuroaxis), artificial

cerebrospinal fluid (aCSF) and drug delivery method (superfusion vs. perfusion), conditions used to elicit gasping, and level of intrinsic neuronal excitability (which is set, in part, by the level of K^+ in the aCSF, see Appendix). Additional studies will be needed to identify the relative importance of these qualities in producing the observed discrepancies.

One notable contradiction in the literature has been the effect of 5-$HT_{2A/2C}$ blockade on gasping in neonatal mouse vs. adult rat *in vitro* preparations. We have for the first time identified a more critical role for endogenous 5-$HT_{2A/2C}$ activation in the hypoxic and gasping responses than previously reported in the arterially-perfused adult rat. Previous reports found that fictive gasping behavior under the influence of KTN, although present and identifiable, was altered in the arterially-perfused rat, while gasps were abolished in the neonatal mouse medullary slice. Again, our observations are in fact more similar to findings from the mouse medullary slice. It is not clear whether primary gasps are truly nonexistent or if the phases of the HVR are merely occurring over a shorter timespan. Additional work from the arterially-perfused adult rat using a specific 5-HT_{2A} receptor antagonist, reported that gasps persisted, but were abolished with blockade of 5-HT_{2A} and NK-1 receptors (148). This suggests that there are additional factors, outside of the 5-HT_{2A} receptor itself that may be closely linked with its role in gasping.

Previous work has yielded some insight into what specifically may be occurring at the post-synaptic level after endogenous 5-HT_{2A} activation. Observations from the mouse medullary slice indicated that fictive eupneic activity is dependent upon the activation of the persistent Na^+ current ($I_{Na(P)}$) and calcium-activated cationic current (CAN) within two populations of pacemaker neurons (cadmium insensitive and sensitive, respectively)(44, 143). Endogenous 5-HT_{2A} activation is required for activation of the cadmium-insensitive pacemaker population, and it appears that this population is critical for generation of gasp-like activity (192). While the data presented in this study suggest that a similar mechanism may be involved in species other than mouse, neither type of pacemaker cells have been identified in the rat to date. This is an important area for future study.

Together, our observations indicate that endogenous and exogenous activation of 5-HT_{2A} receptors influence eupneic breathing, but that endogenous activation of 5-HT_{2A} may play a more critical role in the HVR in the relatively more intact and mature rodent than previously thought. Further studies are needed to clarify whether it is gasping, or the timing of gasping and the HVR that has been abolished under 5-HT_{2A} R blockade, as well as to better understand species related differences in the 5-HT system, including differences in subtype expression, and concurrent post-synaptic mechanisms involved in generation of respiratory activity.

FIGURE III.1

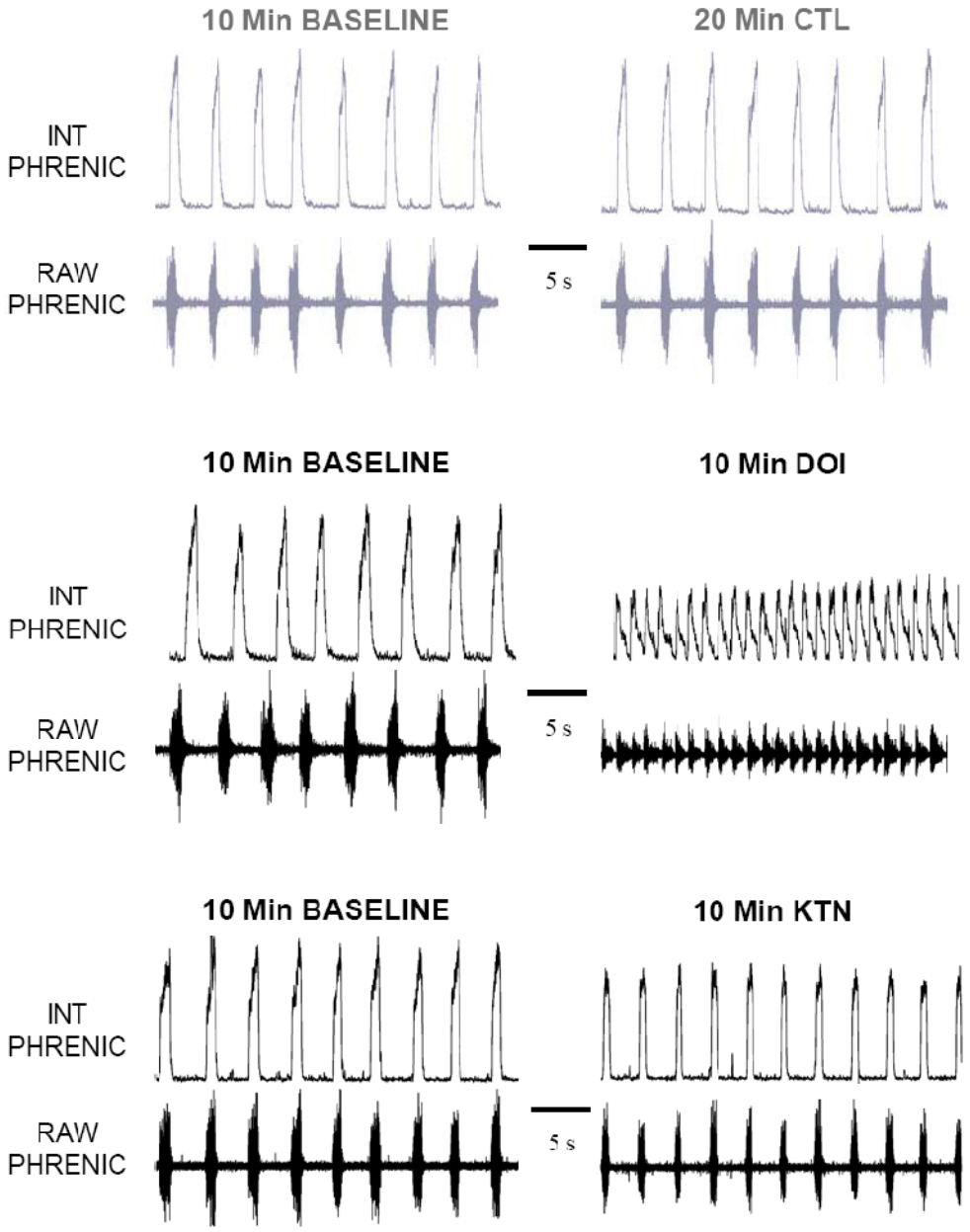

10 Min BASELINE
20 Min CTL
INT PHRENIC
RAW PHRENIC
5 s
10 Min BASELINE
10 Min DOI
INT PHRENIC
RAW PHRENIC
5 s
10 Min BASELINE
10 Min KTN
INT PHRENIC
RAW PHRENIC
5 s

FIGURE III.2

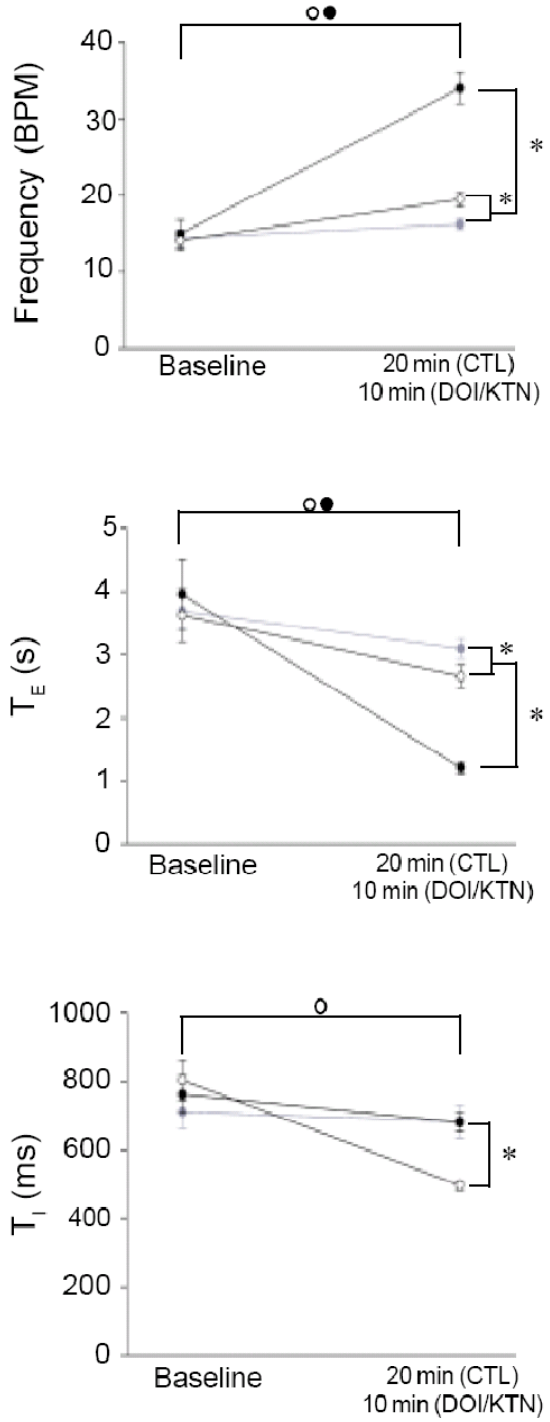

Frequency (BPM)
40
30
20
10
0
Baseline
20 min (CTL)
10 min (DOI/KTN)
*
*
T_E (s)
5
4
3
2
1
0
Baseline
20 min (CTL)
10 min (DOI/KTN)
*
*
T_I (ms)
1000
800
600
400
200
0
Baseline
20 min (CTL)
10 min (DOI/KTN)
*

FIGURE III.3

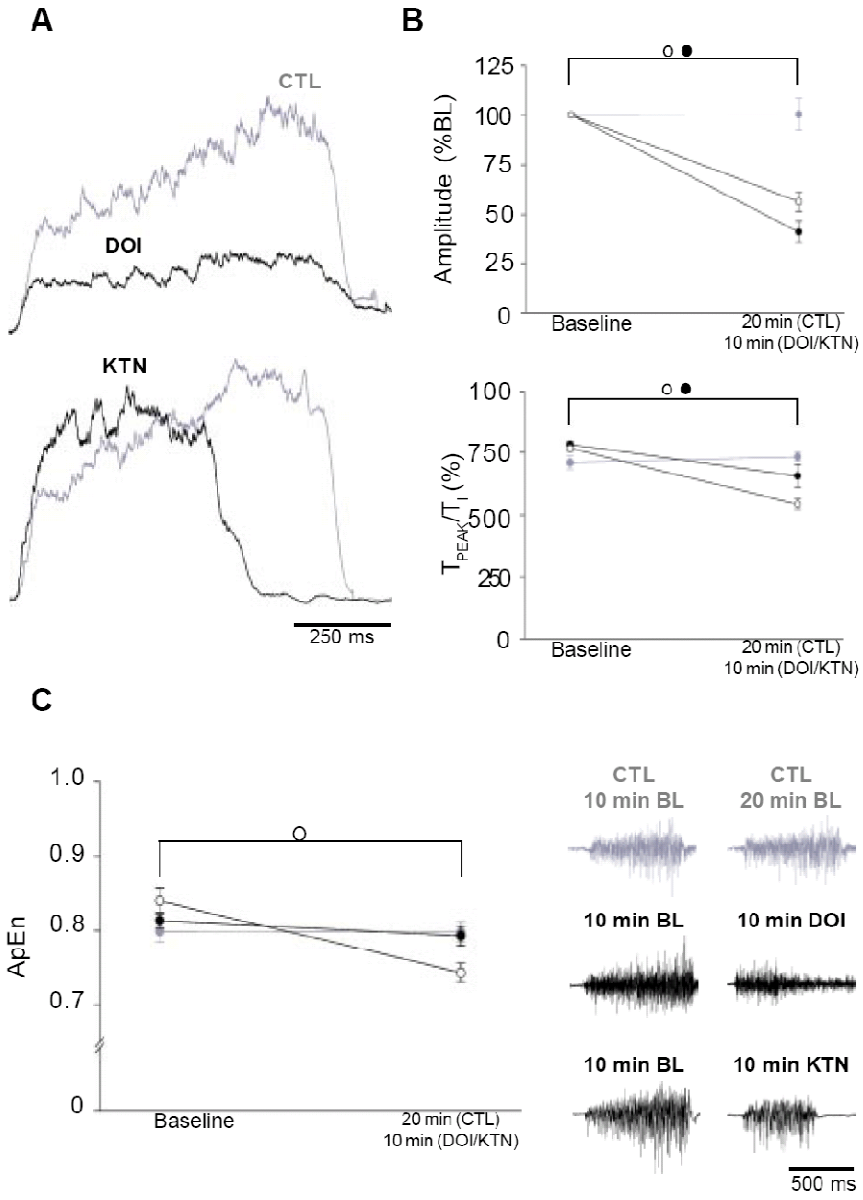

A
CTL
DOI
KTN
250 ms
B
Amplitude (%BL)
125
100
75
50
25
0
Baseline
20 min (CTL)
10 min (DOI/KTN)
T_{PEAK}/T_I (%)
100
750
500
250
0
Baseline
20 min (CTL)
10 min (DOI/KTN)
C
ApEn
1.0
0.9
0.8
0.7
0
Baseline
20 min (CTL)
10 min (DOI/KTN)
CTL
10 min BL
CTL
20 min BL
10 min BL
10 min DOI
10 min BL
10 min KTN
500 ms

FIGURE III.4

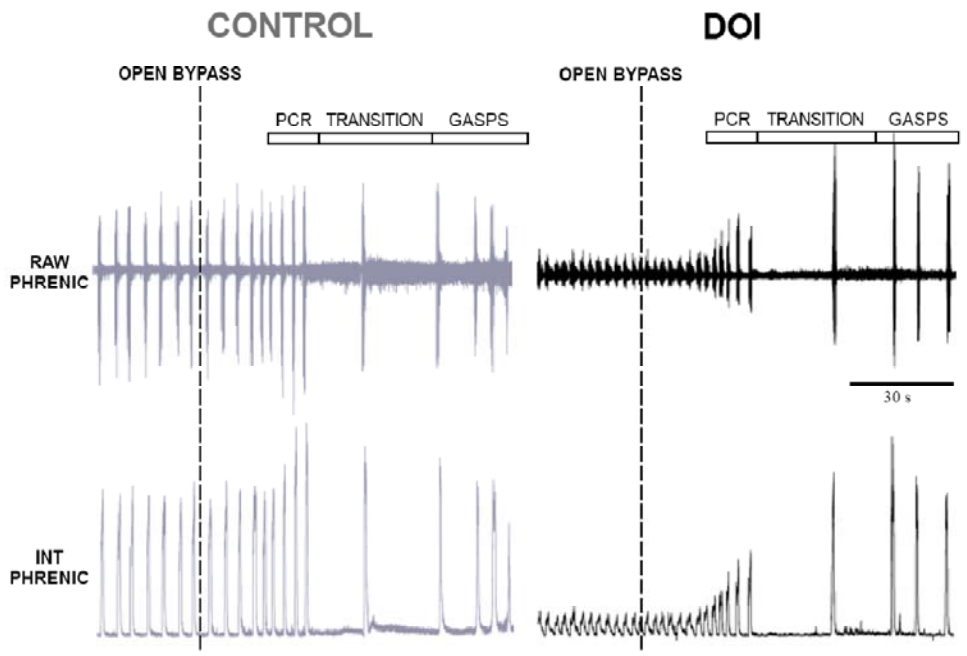

CONTROL
DOI
OPEN BYPASS
OPEN BYPASS
PCR
TRANSITION
GASPS
PCR
TRANSITION
GASPS
RAW
PHRENIC
30 s
INT
PHRENIC

FIGURE III.5

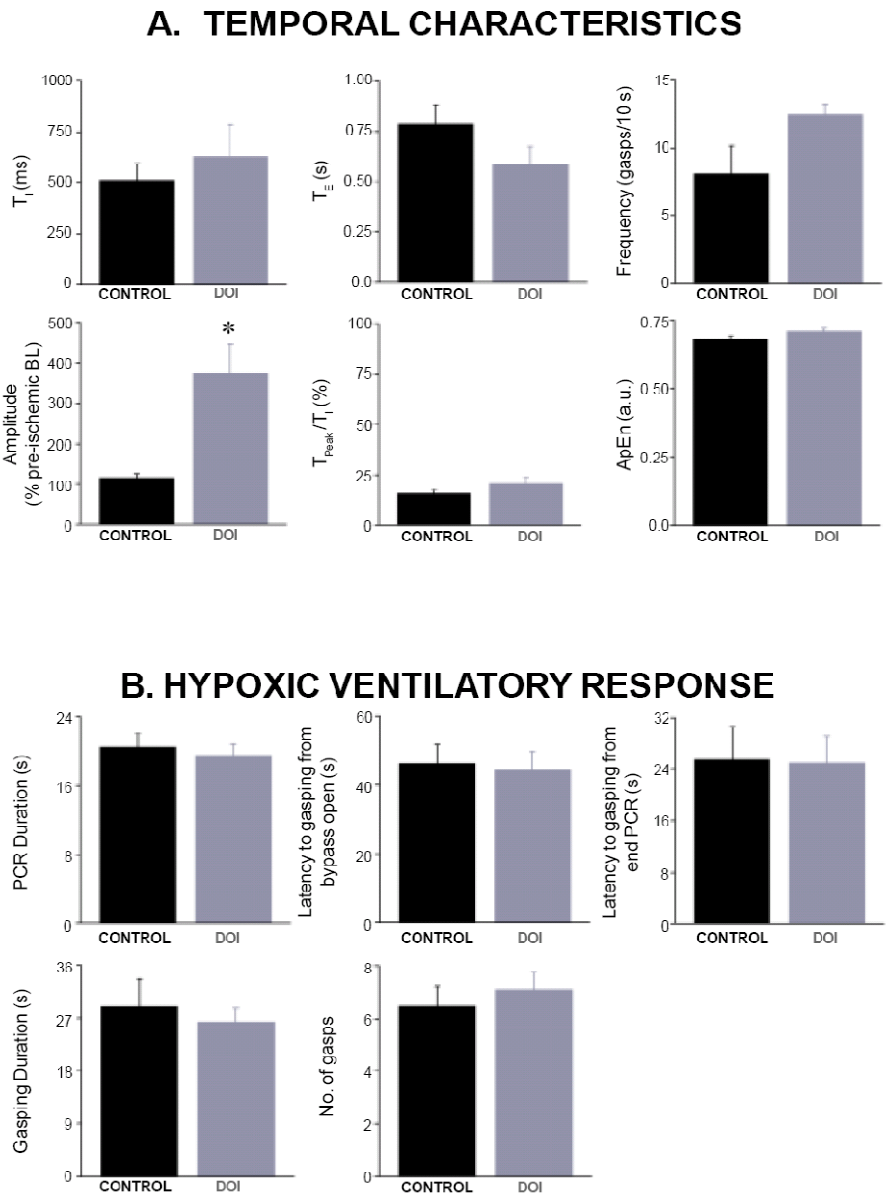

A. TEMPORAL CHARACTERISTICS
T_I (ms)
T_E (s)
Frequency (gasps/10 s)
Amplitude (% pre-ischemic BL)
T_{Peak}/T_I (%)
ApEn (a.u.)
CONTROL
DOI
*
B. HYPOXIC VENTILATORY RESPONSE
PCR Duration (s)
Latency to gasping from bypass open (s)
Latency to gasping from end PCR (s)
Gasping Duration (s)
No. of gasps

FIGURE III.6

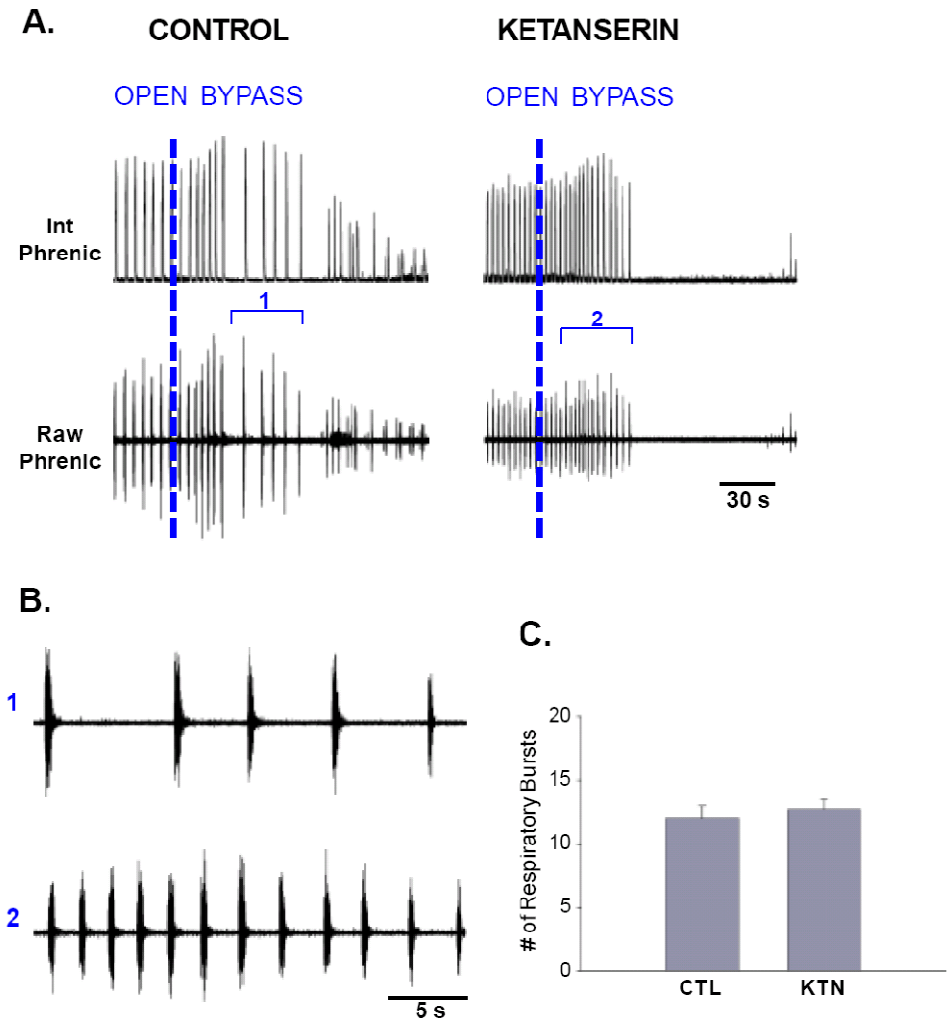

A.
CONTROL
KETANSERIN
OPEN BYPASS
OPEN BYPASS
Int Phrenic
Raw Phrenic
1
2
30 s
B.
1
2
5 s
C.
# of Respiratory Bursts
20
15
10
5
0
CTL
KTN

Figure Legends

Figure III.1. Effects of KTN and DOI on basal phrenic nerve discharge. Examples of raw and integrated phrenic nerve discharge during baseline andperfusion with DOI (middle panel), or with KTN (bottom panel). Both 5-$HT_{2A/2C}$R blockade and activation result in a loss of burst amplitude with respiratory excitation.

Figure III.2. Effects of KTN and DOI on basal phrenic nerve discharge. Summary of temporal changes (frequency, T_I, and T_E) for control(n=12), DOI(n=11), and KTN(n=8). Both DOI (●) and KTN (○) effect reductions in phrenic burst amplitude and increases in burst frequency, however under DOI, T_E is reduced while T_I remains unchanged. Meanwhile under KTN, both T_I and T_E fall significantly to produce the change in frequency. Symbols (● or ○) above brackets indicates significant change from baseline ($P<0.05$) for DOI and KTN, respectively. * indicates significant difference ($P<0.05$) between groups.

Figure III.3. Changes in burst patterning and complexity under perfusion with DOI or KTN. A) Expanded integrated traces of single phrenic bursts under control conditions, or with DOI/KTN. B) Summary data of burst characteristics. Both DOI (●) and KTN (○) effect reductions in phrenic burst amplitude as well as produce a change in burst patterning evident in the fall in T_{PEAK}/T_I. C) Summary of changes in burst complexity. Only KTN results in a significant reduction in burst complexity (ApEn). Symbols (● or ○) above brackets indicates $P<0.01$ for DOI and KTN, respectively.

Figure III.4. Effects of DOI on the hypoxic ventilatory response. Examples of raw and integrated phrenic nerve discharge from two experiments, (time control and DOI) at the onset of ischemia. The phases of the hypoxic ventilatory response are evident with both conditions: peripheral chemoreflex (PCR), transitional phase, and gasping.

Figure III. 5. Effects of DOI on timing and burst characteristics during the hypoxic ventilatory response including gasping (unpaired). A) Gasps elicited by ischemia under DOI appear minimally different from control gaps, except in gasp amplitude. (Note: gasp amplitude is normalized to pre-ischemic baseline bursts. Pre-ischemic bursts under DOI are significantly lower in amplitude compared to controls. When DOI-influenced gasps are normalized to pre-drug, pre-ischemic bursts, no significant difference in gasp amplitude is detected). B)The timing of the phases of the HVR, as well as the number of gasps elicited is unchanged under DOI.

Figure III.6. Effects of KTN on the hypoxic ventilatory response (paired). Examples of raw and integrated phrenic nerve discharge, before and after the application of KTN, at the onset of ischemia (open bypass, A). Phases of the HVR including gasping are lost. However expanded raw traces (shown in B) of control gasps (1) and bursts occurring late in the response under KTN (2), reveal that the bursts retain many of the typical characteristics of gasps (i.e.; shortened duration and decrementing pattern). C) The number of phrenic bursts elicited after the peak of the PCR is unchanged under KTN.

CHAPTER IV

SPECIFIC AIM 2

The effects of endogenous 5-HT_{2A} receptor blockade on the ventilatory response to increased CO_2 (hypercapnia).

Introduction

A number of pathologies of breathing are postulated to arise from the failure to sense or respond appropriately to elevated levels of CO_2 (hypercapnia), and accumulating evidence suggests that disturbances in the serotonergic system may contribute to their etiology. For many years, studies have uncovered important modulatory roles for serotonin (5-HT) in various aspects of central respiratory control. Serotonergic raphe neurons have been identified as putative respiratory CO_2 chemosensors, yet the role of endogenous serotonin (5-HT) in the sensation of and response to elevated levels of CO_2 is poorly understood. Recent investigations in adult rats *in vivo*, however, have demonstrated that destruction of raphe serotonergic neurons by a saporin-serotonin transporter conjugate decreases the ventilatory response to increased inspired CO_2 (125) while excess endogenous 5-HT produced by chronic microdialysis of the selective 5-HT reuptake inhibitor fluoxetine increases the ventilatory response to CO_2 (184).

The activation of chemosensitive 5-HT neurons in response to rising levels of CO_2 presumably leads to release of 5-HT, which in turn acts on 5-HT receptors to produce respiratory excitation. Additional studies have begun to identify roles for activation of various 5-HT receptors in central CO_2 chemoreception. Specifically, blockade of the excitatory 5-HT_{2A} receptor in cultured embryonic rat brainstem neurons has been shown to markedly reduce neuronal responses to elevated levels of CO_2 (182). However, a role for the 5-HT_{2A} receptor in the ventilator response to elevated CO_2 requires clarification. Therefore, the current study was undertaken to evaluate the effects of blockade of 5-HT_{2A} receptors on the hypercapnic ventilatory (phrenic) response in an arterially-perfused adult rat preparation.

Since the excitatory effects of 5-HT on breathing may be accounted for by 5-HT_{2A} receptor activation, we hypothesized that blockade of 5-HT_{2A} receptors would disrupt the characteristic ventilatory response to elevated levels of CO_2.

Methods:

All experiments were performed in the arterially-perfused adult rat preparation was used (n=33). The methods and procedures for this experimental preparation have previously been discussed in detail, and will not be repeated here (see General Methods).

In these experiments phrenic nerve discharge was continuously recorded before and during a 5% CO_2 challenge, while perfusing with aCSF containing K^+ as stated (no drug, control conditions). Baseline data was acquired while the preparation was perfused with aCSF gassed with 95% O_2/5% CO_2 for a minimum of 10 minutes. The gas bubbling the aCSF was then changed to 90% O_2/10% CO_2 (a 5% CO_2 hypercapnic challenge) for 5 minutes, after which the gas was returned to baseline levels. The preparation was allowed to recover for 10 minutes at which point, KTN (40 μM) was added to the perfusate. Ten minutes were allowed for the drug to exert an effect, before the hypercapnic challenge was repeated. This allowed for a 20 minute recovery period between hypercapnic trials, and 10 minutes for the effects of KTN to reach steady-state (see Aim 1). Since various experimental preparations utilize different levels of extracellular K^+ ($[K^+]_o$), the role of endogenous 5-HT acting on the 5-HT2A receptor in hypercapnia was also evaluated for these experiments at three levels of $[K^+]_o$: 3.0 mM K^+, similar to "normal" physiological levels (n=6); 4.25 mM K^+, as occasionally used in this preparation (n=7); and 6.25 mM K^+, the typical level of $[K^+]_o$ used in this preparation (n=6).

Control Experiments:

A series of time control experiments (n=14) were also conducted in each $[K^+]_o$ of interest: of 3mM (n=5), 4.25mM (n=4), and 6.25 mM (n=5). In these experiments phrenic nerve discharge was continuously recorded before and during a 5% CO_2 challenge, with aCSF of $[K^+]_o$ as stated, containing no drug.

Baseline data were recorded while the preparation was perfused with aCSF gassed with 95% O_2/5% CO_2 for a minimum of 10 minutes. The gas bubbling the aCSF was then changed to 90% O_2/10% CO_2, for 5 minutes, after which the gas was returned to baseline levels. Recovery was allowed for at least 20 minutes before repeating the 5% hypercpanic trial. (Summary data is presented in Fig.IV.6)

Data Acquisition and Analysis: (See *General Methods*)

Temporal Analyses:

Individual traces of time series data from the PowerLab recordings were output for temporal analyses. Inspiratory burst duration (T_I), the duration between bursts (T_E), burst frequency, burst amplitude, and the time-to-peak (T_{peak}) were determined for basal phrenic nerve discharge (baseline and recovery) and phrenic nerve activity during hypercapnia. Burst amplitude data was normalized to baseline levels of discharge, which was set to 100% in each preparation, and T_{peak} was normalized to T_I (T_{peak}/T_I, expressed as a percentage). Average values for these variables were calculated from 10 consecutive respiratory cycles during the last minute of each condition, when the preparation reached steady-state.

Approximate Entropy Analyses: The complexity of the phrenic nerve discharge was quantified using *approximate entropy* (ApEn) analysis (see General Methods). For an individual experiment, ApEn was calculated for each burst

within 10 consecutive respiratory cycles, during the last minute of each condition, and averaged over the 10 bursts for each condition.

Statistical Analyses: (See General Methods) The use of a paired paradigm has allowed us to evaluate each experiment as its own control. Time control studies were conducted using a separate series of experiments.

Results

General effects of hypercpania on phrenic nerve discharge at three levels of $[K^+]_o$:

At each level of $[K^+]_o$ hypercapnia elcicited respiratory excitation with respiratory frequency increasing ~20-30% over baseline fregquency. This increase in frequency was mediated by both a reduction in T_I ($P<0.05$) and T_E ($P<0.05$). In most cases CO_2 also caused an increase in phrenic burst amplitude. All changes were reversible within ten minutes of recovery from the CO_2 challenge (Fig. IV.1).

General effects of Ketanserin on temporal characteristics and complexity of basal phrenic nerve discharge:

As previously demonstrated, KTN modified basal (eupneic) phrenic nerve discharge: these effects are summarized in Fig.IV.2 for each level of $[K^+]_o$ examined. In all experiments, perfusion with KTN reduced phrenic burst ampliture, and increased phrenic burst frequency. The reduction in burst

amplitude corresponded to ~25-40%, while the increase in frequency corresponded to ~30-50%. Both T_I and T_E were decreased ($P<0.01$) a similar magnitude of response was observed regardless of the level of $[K^+]_o$ examined. Burst patterning was altered from an augmenting discharge pattern to a more bell-shaped burst. In addition to these temporal effects, KTN reduced ApEn at each level of $[K^+]_o$.

Effects of Ketanserin on the hypercpaninc ventilatory response:

At each level of $[K^+]_o$, hypercapnia increased phrenic nerve discharge in a similar manner to that observed under control conditions. Specifically, during perfusion with KTN, hypercapnia elicited a robust increase in phrenic burst frequency (Fig IV.3). Summary data of all parameters studies at each level of $[K^+]_o$ are provided in Table IV.1. However all changes under hypercapnia occurred on the background of changes in basal phrenic nerve discharge as noted in Fig. IV.2. Therefore, in order to assess the magnitude of these changes, all data obtained during the hypercapnic trials were normalized to pre-hypercpanic baseline levels in figure IV.3.

The tendency for burst amplitude to increase during hypercapnia is preserved under KTN conditions, regardless of the level of $[K^+]_o$. It should be noted that under both control and KTN conditions, the increase in amplitude is small and failed to reach significance.

The hypercapnia-induced increase in frequency was of similar magnitude during perfusion with KTN as under control conditions (~120-140% of basal frequency) at each level of $[K^+]_o$. Furthermore, under both conditions, T_E is significantly shortened in duration, in a manner independent of $[K^+]_o$. The contribution of T_I to the hypercapnic frequency response however, is markedly different between control and KTN conditions. Under control conditions, the hypercapnia-induced increase in frequency is is mediated by reductions in both T_E and T_I. However, under the influence of KTN, T_I fails to shorten in response to elevated CO2. Under control conditions, T_I during hypercapnia was reduced by ~15-25% from baseline values. Meanwhile, during perfusion with KTN, T_I was slightly (albeit not significantly) elevated over KTN baseline values at all three levels of $[K^+]_o$.

Hypercapnia under both control and KTN conditions elicited a significant change in patterning across levels of $[K^+]_o$ (Fig. IV.4). Peak activity shifted to an earlier timepoint within the burst (~50-70% of baseline values), however KTN itself was demonstrated to induce a patterning change during basal activity (Fig IV.2), whereby the burst shape changed from augmenting to bell shaped. While the magnitude of the shift in peak activity to earlier with the phrenic burst remains equivalent between KTN and control conditions, peak activity is shifting from different positions within the burst (Fig. IV.4)

Burst complexity appeared to be the only parameter studied in which the level of $[K^+]_o$ made any significant contribution. This was also evident during time control experiments (Fig.IV.6). The first hypercapnic trial in 3mM $[K^+]_o$, (i.e. control) was not accompanied by a change in ApEn (P=0.37), while with KTN the hypercapnic trial is assosciated with an increase in ApEn (P=0.002, in stark contrast to time control observations, wherein the second hypercapnic trial was assosciated with a reduction in ApEn). A similar trend was observed in 4.25 mM $[K^+]_o$, where the first/control hypercapnic trial produced a reduction in ApEn (P=0.004). However under KTN hypercapnia produced a significant increase in ApEn (P=0.004). At 6.25 mM$[K^+]_o$, changes in ApEn from baseline to hypercapnia failed to reach significance under either condition (Control:P=0.11, KTN: P=0.12). However, at all levels of $[K^+]_o$ the change in ApEn sustained under KTN was significantly different from that under control conditions ($P<0.05$).

Discussion

The data presented in this study demonstrate that a ventilatory response to elevated CO_2 can be elicited under KTN-induced blockade of 5-HT_{2A} receptors. This response is comparable in magnitude, in terms of amplitude, frequency, and degree of patterning change, however differences exist in both the manner in which the increase in breathing frequency is mediated (T_I/T_E), as well as in the accompanied changes in burst complexity. Our data indicate that while under

control conditions, burst duration and time between bursts decrease, the shortening of T_I under KTN is maximal. These changes appeared to occur largely independent of K^+, excepting burst complexity.

In contrast to findings from cultured medullary neurons (182), in preparations where a relatively intact neuroaxis is present, such as the one utilized here, 5-$HT_{2A/2C}$ receptor activation does not appear to be critical to form a response to hypercapnia. However, 5-$HT_{2A/2C}$ receptor activation does seem to play some role in the normal HCVR. To our knowledge, this study represents the first attempt to address the contribution of endogenous 5-HT_{2A} receptor activation in the establishment of the ventilatory response to hypercapnia, in this preparation.

It is to be noted that in this study, as in previous reports from our lab and others, blockade of 5-$HT_{2A/2C}$ receptors via ketanserin resulted in an increase in frequency (excitation) although amplitude was reduced. Several possible explanations for this exist, including 1) blocking the activity of 5-HT neurons projecting to inhibitory neurons (i.e. GABAergic) to produce loss of inhibition and thereby unmasking respiratory excitation(25) 2) the persistent activity of additional excitatory molecules co-released with 5-HT (i.e. Substance P and TRH) (148) 3)reciprocal function of additional neurotransmitter systems, i.e. norepinephrine (197).

The present experiments also addressed the effect of different levels of extracellular K^+ in the establishment of the HCVR in *in vitro* preparations, with and without the influence of KTN. While our data suggests that extracellular potassium at levels utilized exerts minimal influence on the effect of KTN on the HCVR, we have not eliminated the possibility that the use of different potassium levels in aCSF could affect the inherent excitability of the preparation. Evaluating the complexity of the signal may be useful in gaining insight into this possibility. Presupposing that $[K^+]_o$ contributes to setting the level of intrinsic excitability of the preparation, then a preparation perfused with 3mM aCSF would be somewhat less excitable than one perfused with 4.25 mM, and both would display a lesser degree of excitability than a preparation perfused with 6.25mM aCSF. Furthermore, the response to elevated CO_2 involves increased synchronization of neuronal elements. A lower ApEn value may also be indicative of increased organization, or synchronization, of phrenic nerve discharge. Our measurement of ApEn, may be affected by two different factors: 1) intrinsic excitability of the preparation and 2) the dynamics of synchronization under the hypercapnic challenge.

At 3mM, fewer neuronal elements are likely to be activated at baseline, therefore the increase in synchronization (drop in ApEn) during the first hypercapnic trial may not be robust enough to reach statistical significance. Meanwhile the hypercapnic challenge itself works to increase excitability, thus, even though there is recovery by the second hypercapnic challenge, the change in

synchronization is now undergone by sufficiently more individual elements to detect the change. Therefore while the trend of ApEn is to fall at 3 mM, during the first hypercapnic trial of the time control experiments (which corresponds to the control trial during the KTN experiments), it fails to reach statistical significance, while the fall during the second control trial is evident. At 4.25, it may be that the level of intrinsic excitation is sufficient to reveal the dynamics of increased synchronization under hypercapnia during both trials. However under 6.25 mM it is possible that there are a far greater number of active elements at the outset. Perhaps this greater degree of intrinsic activity is saturating the increase in synchrony occurring under hypercapnia.

KTN, at all levels of K^+ resulted in reduced ApEn, or a higher degree of ordered elements within the bursts. However, while under the influence of KTN, hypercapnia failed to result in further reductions in entropy. Rather there is an increase in entropy over baseline KTN values at both 3 mM and 4.25 (while under control conditions, this trial was associated with significant reductions in ApEn). While the increase fails to reach detection under 6.25 as compared to baseline, the magnitude of change between control and KTN-influenced hypercapnic trials is significantly different for all levels of K+. The loss of endogenous 5-$HT_{2A/2C}$ modulation may result in a loss of neuronal units that are normally recruited in a synchronous manner to respond to elevations in CO_2. Therefore, under the influence of KTN, additional and atypical units are potentially recruited to mount

the response to hypercapnia, however these additional elements may not be ideally synchronous with the rest of the network.

Prior identification of serotonergic neurons as chemosensors, in conjunction with evidence to suggest that selectively activating or blocking specific 5-HT receptor subtypes may alter the ventilatory response to hypercapnia, suggested that 5-HT exerts an important modulatory influence on the hypercapnic ventilatory response. As responding to elevated CO_2 involves respiratory excitation, and 5-HT_{2A} activation had previously been demonstrated to account for the excitatory effects of elevated 5-HT on breathing, and previous work has ascribed an important role for endogenous 5-HT_{2A} activation in formation of the normal hypoxic ventilatory response (11, 144, 176) it appeared likely that endogenous 5-HT_{2A} activation might supply a critical component of the HCVR.

On the whole, however, our observations indicate that while loss of endogenous 5-HT_{2A} activation alters the hypercapnic ventilatory response to a certain, albeit mild, extent, the HCVR remains largely intact. These findings do not necessarily oppose previous work suggesting the importance of 5-HT neurons as putative chemosensors. Although the 5-HT_{2A}R is extensively expressed in many of the major respiratory nuclei, additional receptor subtypes exist, including the 5-HT_4 and 5-HT_7 subtype. These function through Gs pathways, leading to excitation of the postsynaptic cell, and have been identified in the region most

likely to serve as the central respiratory pattern generator, the pre-Bötzinger complex(92, 156). To date, little is known about the functional roles of these receptors in respiratory behavior. Investigating their role in generating the hypercapnic ventilator response represents an important area for future work.

Additionally, little is known about the post-synaptic intracellular effects of downstream of 5-HT activating it's various receptors in the context of hypercapnia. Future work will also need to identify specifically which channels and currents are being affected by 5-HT neuromodulation (i.e. TASK channels, CAN currents). Furthermore, while it is possible that additional neurotransmitter/chemosensory pathways may be accounting for the hypercapnic ventilatory response in the absence of 5-HT_{2A} activation via an independent mechanism, it is also possible that the pathways may converge at the level of the intracellular effector system (i.e. elevated cAMP and intracellular Ca^{+}). Clarification of these mechanisms will help to further our understanding of this vital and important homeostatic function.

Figure IV.1

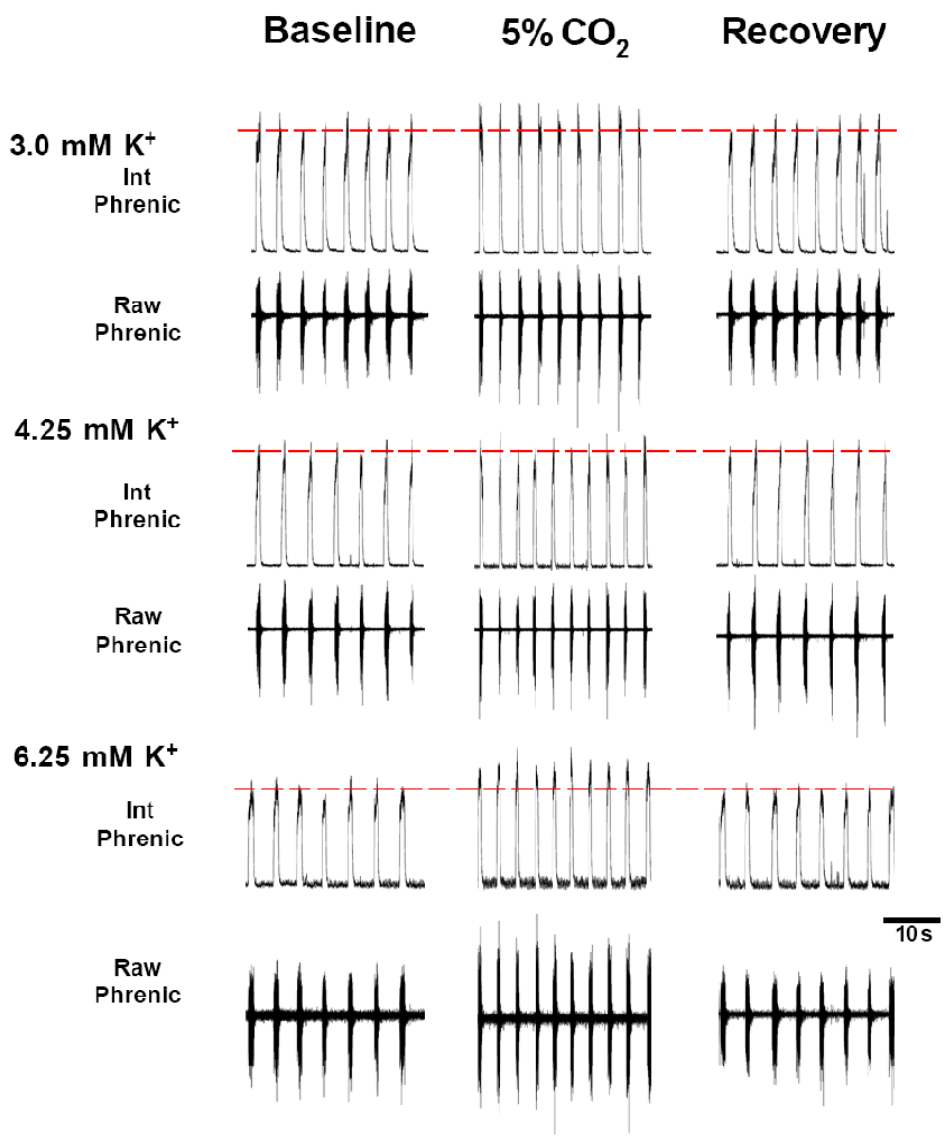

Baseline
5% CO_2
Recovery
3.0 mM K^+
Int Phrenic
Raw Phrenic
4.25 mM K^+
Int Phrenic
Raw Phrenic
6.25 mM K^+
Int Phrenic
Raw Phrenic
10 s

Figure IV.2

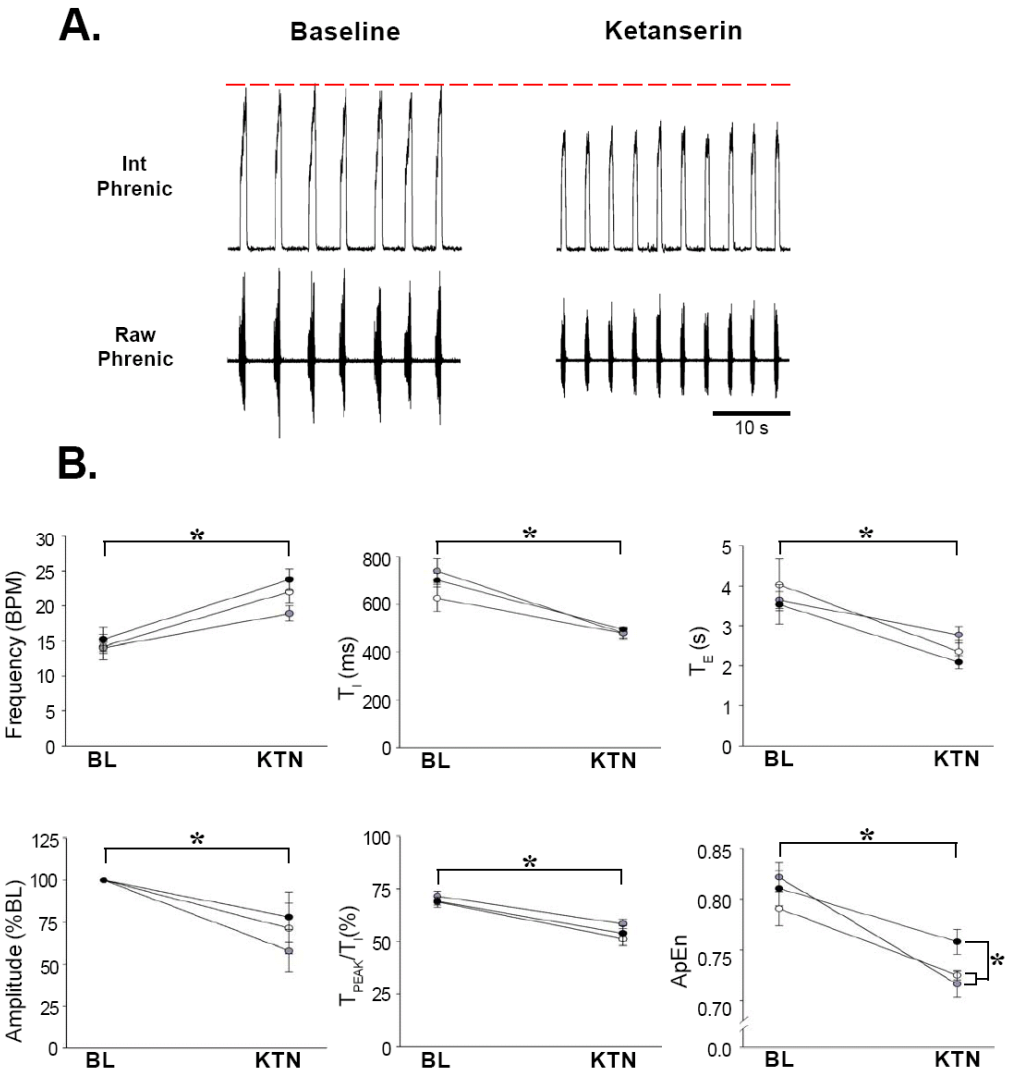

A.
Baseline
Ketanserin
Int Phrenic
Raw Phrenic
10 s
B.
Frequency (BPM)
T_I (ms)
T_E (s)
Amplitude (%BL)
T_{PEAK}/T_I(%)
ApEn
BL
KTN
*

Table IV.1

		Control			Ketanserin		
		Baseline	*CO_2*	*Recovery*	*Baseline*	*CO_2*	*Recovery*
	$[K^+]_o$						
T_I	3.0 mM	626.9±57.3	521.3±13.5 *	583.8±29.6	477.5±19.7 §	512.1±12.0	545.7±16.3
(ms)	4.25 mM	741.1±49.8	553.1±31.4 *	763. 5±43.2	478.5±24.8 §	496.6.3±27.6	579.4±42.2 §
	6.25 mM	701.9±29.1	579.4±22.4 *	674.9±29.5	495.6±9.8 §	516.7±16.9	539.7±11.9 §
T_E	3.0 mM	4.0±0.7	3.2±0.3 *	3.5±0.6	2.4±0.3 §	1.8±0.2 §*	2.0±0.2 §
(s)	4.25 mM	3.6±0.2	2.9±0.2 *	3.5±0.2	2.8±0.2 §	1.6±0.2 §*	2.1±0.1 §
	6.25 mM	3.5±0.5	2.5±0.2 *	3.4±0.7	2.1±0.2 §	1.6±0.1 §*	2.3±0.3 §
Frequency	3.0 mM	14.2±1.8	16.8±1.4 *	15.3±2.1	22.0±1.7 §	27.4±2.4 §*	24.1±2.0 §
(breaths/min)	4.25 mM	14.0±0.8	17.8±0.9 *	14.1±0.4	18.9±1.1 §	29.2±2.0 §*	22.9±0.4 §
	6.25 mM	15.2±1.7	20.2±1.4 *	16.9±2.4	23.8±1.5 §	29.3±1.9 §*	22.3±1.8 §
Amplitude	3.0 mM	100±0.0	125.3±15.5	116.9±13.5	71.1±15.0 §	75.3±16.1 §	64.8±14.4 §
(% BL)	4.25 mM	100±0.0	112.7±3.8	92.0±6.1	57.8±12.7 §	63.6±8.3 §	52.6±5.5 §
	6.25 mM	100±0.0	125.0±13.2	117.6±1.7	77.9±14.9 §	76.9±18.1 §	76.4±18.4 §

Figure IV.3

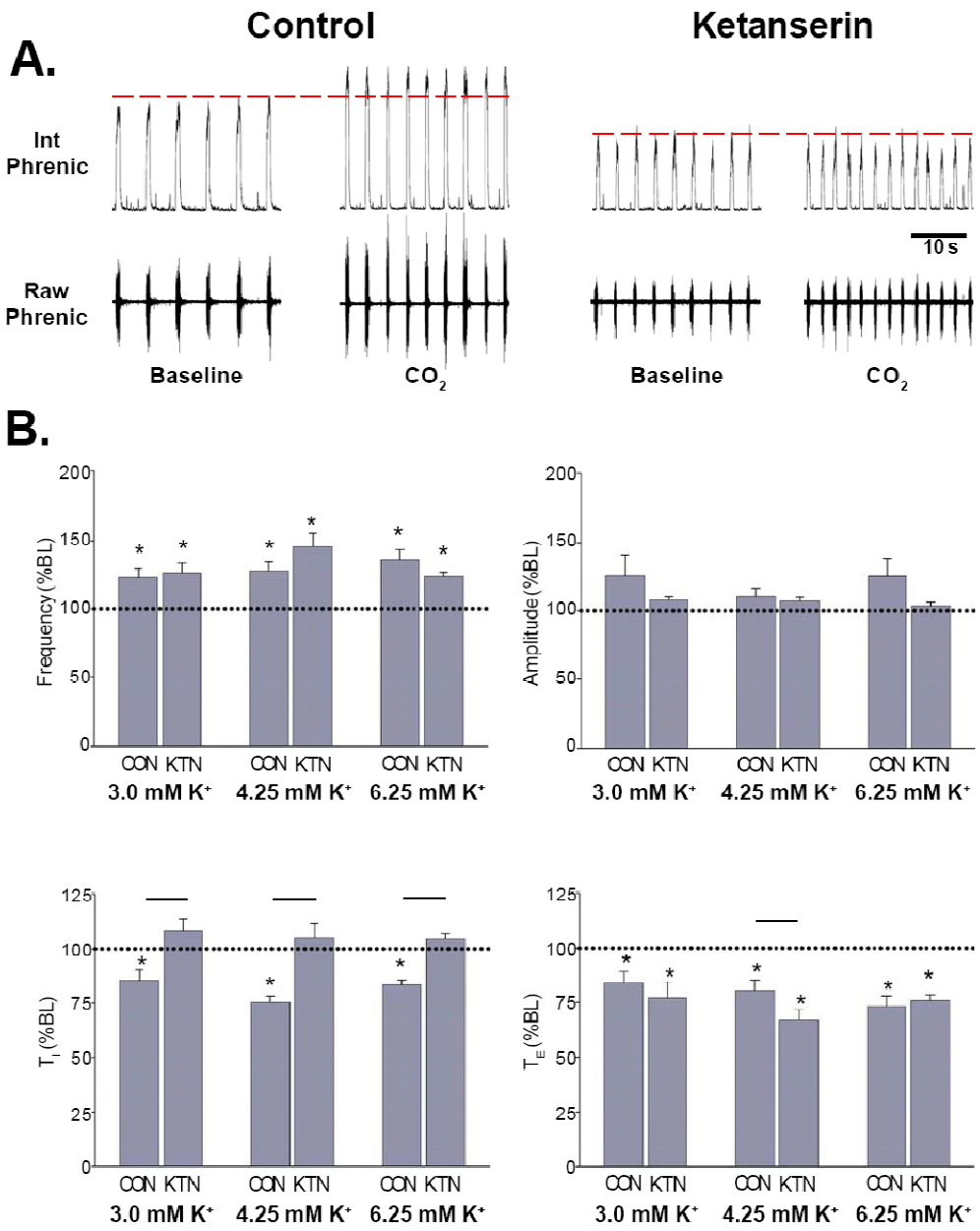

A.
Control
Ketanserin
Int Phrenic
Raw Phrenic
10 s
Baseline
CO2
Baseline
CO2
B.
Frequency (%BL)
Amplitude (%BL)
CON KTN
3.0 mM K+
4.25 mM K+
6.25 mM K+
TI (%BL)
TE (%BL)

Figure IV.4

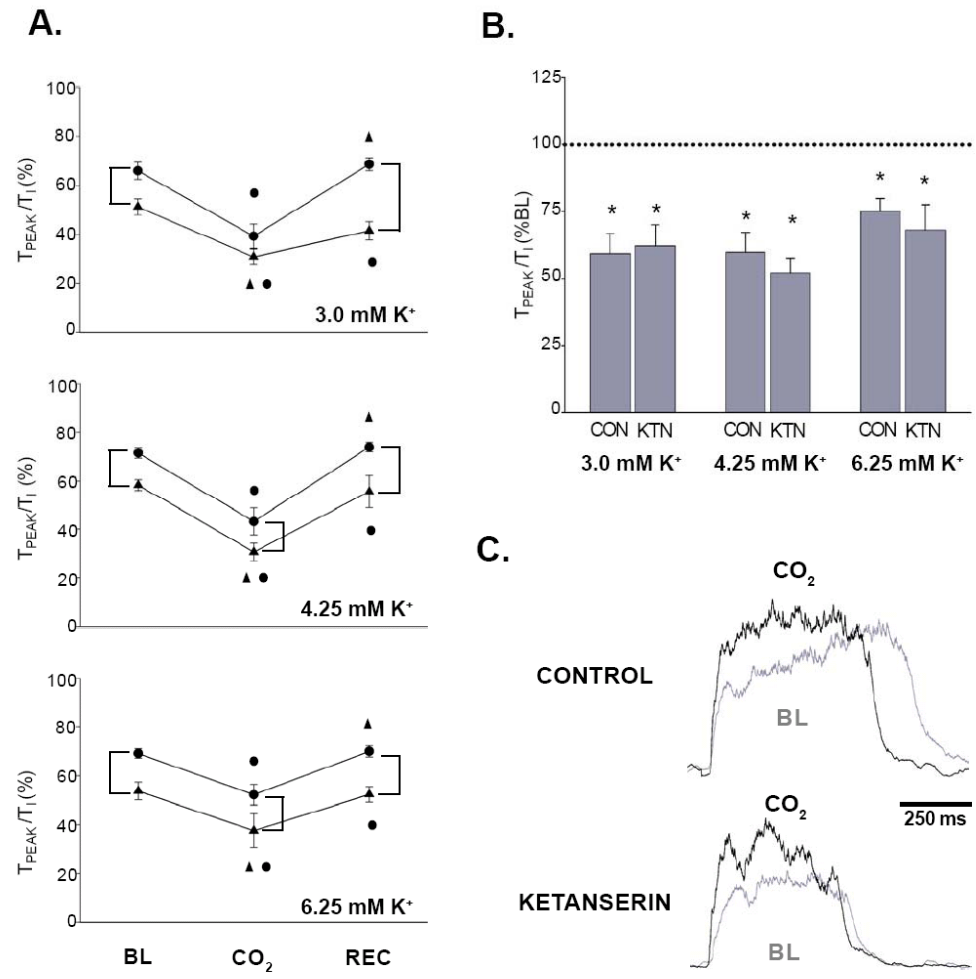

A.
B.
C.
T_{PEAK}/T_I (%)
3.0 mM K+
4.25 mM K+
6.25 mM K+
BL
CO_2
REC
T_{PEAK}/T_I (%BL)
CON KTN
CONTROL
KETANSERIN
250 ms

Figure IV.5

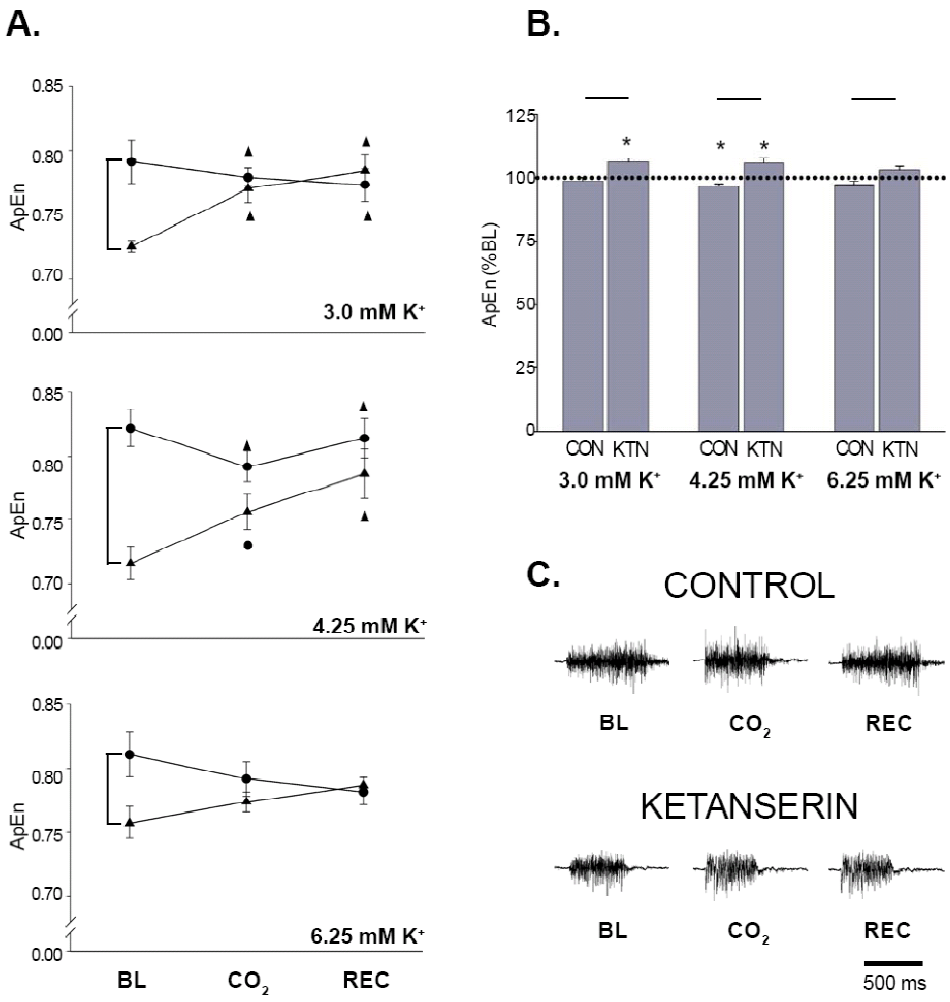

A.
B.
C.
ApEn
0.85
0.80
0.75
0.70
0.00
3.0 mM K+
4.25 mM K+
6.25 mM K+
BL
CO2
REC
ApEn (%BL)
125
100
75
50
25
0
CON KTN
CON KTN
CON KTN
3.0 mM K+
4.25 mM K+
6.25 mM K+
CONTROL
KETANSERIN
500 ms

Figure IV.6

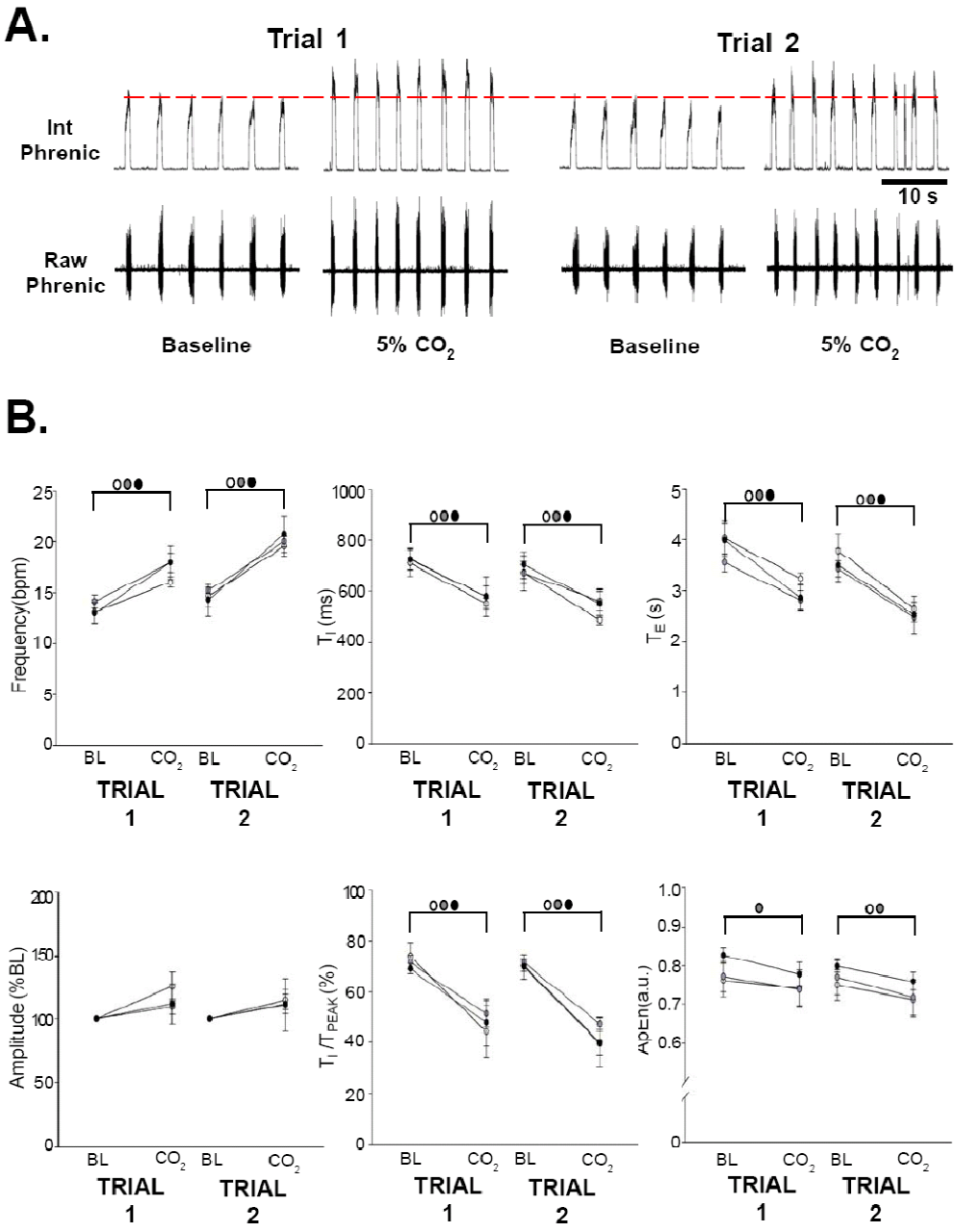

A.
Trial 1
Trial 2
Int Phrenic
Raw Phrenic
10 s
Baseline
5% CO2
Baseline
5% CO2
B.
Frequency(bpm)
TI (ms)
TE (s)
Amplitude (%BL)
TI /TPEAK (%)
Apnea(a.u.)
BL
CO2
TRIAL 1
TRIAL 2

Figure Legends

Figure IV.1 . Effects of CO_2 on phrenic nerve activity in the arterially perfused adult rat at three levels of $[K^+]_o$. Examples of phrenic nerve activity recorded during three different experiments.

Figure IV.2. Effects of KTN on phrenic nerve activity in the arterially perfused adult rat. (A) Example of phrenic nerve discharge during eupnea under control conditions and the influence of KTN. (B) Summary data showing timing, patterning, and complexity of phrenic nerve discharge during eupnea during control (CON) conditions and perfusion with Ketanserin (KTN) at 3.0 (open circle, n=6), 4.25 (gray circle, n=7) and 6.25 (black circle, n=6) mM $[K^+]_o$. Application of KTN reduced phrenic burst amplitude, and increased phrenic burst frequency. Phrenic burst pattern was altered from augmenting to a bell-shaped burst, as reflected by the shift in T_{PEAK}. KTN reduced burst complexity. * denotes significant difference ($P<0.05$, as determined by paired T-test within each $[K^+]_o$ level).

Table IV.1. Changes in temporal characteristics and burst amplitude with hypercapnia, under control and KTN conditions. Summary of mean (±S.E.) values of temporal data (T_I, T_E, Frequency) and amplitude, at 3.0 (n=6), 4.25 (n=7), and 6.25mM $[K^+]_o$, (n=6). Asterix (*) indicates significant change from pre-hypercapnic baseline, while significant differences between corresponding timepoints (baseline, CO_2, recovery) during control conditions versus under the influence of KTN are indicated by § ($P<0.05$, as determined by Two-Way Repeated Measures ANOVA).

Figure IV.3. Effects of KTN on the magnitude of the hypercapnic ventilatory response. (A) Example demonstrating phrenic nerve discharge (at 6.25mM $[K^+]_o$), before and during a 5% hypercapnic challenge, under control and KTN conditions. (B) Summary data showing changes in temporal characteristics and burst amplitude normalized to pre-hypercapnic baseline (100%, indicated by dashed line) , before and during perfusion with KTN. *denotes a significant change from baseline, black bar indicates a significant difference as indicated ($P<0.05$). T_I is significantly reduced by hypercapnia under control conditions but not under KTN. Reductions in T_E, and increases in burst frequency and amplitude are equivalent in magnitude under both conditions.

Figure IV.4. Effects of KTN on changes in phrenic burst pattern during hypercapnia. (A)Summary data showing patterning changes during baseline (BL), 5% CO_2, and recovery (REC), before (control, ●) and during perfusion with KTN(▲) at 3.0 (n=6), 4.25 (n=7), and 6.25mM $[K^+]_o$, (n=6). Symbols (●,▲) denote significant change from control or KTN-influenced BL, respectively while brackets indicate a significant difference as indicated ($P<0.05$, determined by One-Way RM ANOVA). CO_2 results in a shift of peak inspiratory activity to an earlier timepoint within the burst. (B) Summary of T_{PEAK}/T_I during hypercapnia,

as normalized to pre-hypercapnic baseline, before and during perfusion with KTN at 3, 4.25, and 6.25mM $[K^+]_o$. The magnitude of the shift in T_{PEAK} is conserved under KTN. * denotes a significant change from eupnea. (C) Example ($[K^+]_o$ =6.25mM) of expanded integrated phrenic bursts during 5% CO_2, overlaid with a typical eupneic burst for the same condition (control or KTN). Eupnea is characterized by an augmenting phrenic burst discharge while CO_2 results in a reversible shift to a more bell-shaped pattern. KTN produces a bell-shaped pattern in eupnea, with CO_2 resulting in a more decrementing pattern of discharge.

Figure 5. Effects of KTN on changes in phrenic burst complexity (ApEn) during hypercapnia. (A)Summary data of changes in burst complexity with hypercapnia, under control conditions (●) and during perfusion with KTN(▲) at (n=6), 4.25 (n=7), and 6.25mM $[K^+]_o$, (n=6). Symbols (●,▲) denote significant change from control or KTN-influenced BL, respectively ($P<0.50$). KTN results in a significant reduction in ApEn. (B) Summary of ApEn during hypercapnia, as normalized to pre-hypercapnic baseline, before and during perfusion with KTN at three levels of $[K^+]_o$. The change in entropy that occurs with hypercapnia is significantly different between control and KTN-influenced conditions at all $[K^+]_o$. Hypercapnia reduced complexity under control conditions (significantly at 4.25 mM $[K^+]_o$). In contrast, with KTN, burst complexity was increased by the addition of hypercapnia (significant at 4.25 mM $[K^+]_o$). (C) Examples of expanded raw phrenic bursts (4.25 mM $[K^+]_o$) before, during, and after a 5% CO_2 trial under control conditions and KTN.

Figure 6. Time-control experiments using arterially perfused adult rat discharge in paired hypercapnic trials **A.** Examples of integrated and raw phrenic nerve discharge before and during paired hypercapnic trials in a single experiment (4.25 mM K^+). Trials were 5 minutes in duration separated by a 20 min recovery period. **B.** Summary data obtained from time control experiments at 3 (open circle, n=5), 4.25 (gray circle, n=4), and 6.25 (black circle, n=5) mM [$K^+]_o$. At all levels of $[K^+]_o$ hypercapnia caused an increase in burst frequency, and change in phrenic burst pattern. Burst amplitude exhibited a tendency to increase under hypercapnic conditions. No significant differences were noted between trials 1 and 2, or among levels of $[K^+]_o$, excepting ApEn where significant reduction under hypercapnia only occurred under both trials at 4.25 mM $[K^+]_o$, and under trial 2 at 3 mM$[K^+]_o$ (determined using One-Way ANOVA with and without Repeated Measures, as appropriate).

CHAPTER V

SPECIFIC AIM 3

The effects of chronically elevated 5-HT on the ventilatory response to hypercapnia in wild-type and Connexin 32-deficient mice.

Introduction:

While 5-HT neurons of the medullary raphe have been strongly suggested to function as central chemosensors, a wide variety of perturbations independent of 5-HT neuromodulation have been shown to alter the hypercapnic ventilatory response (HCVR). In fact, little is known about how 5-HT neurons function within the intact neural network alongside a number of other pH-sensors to mediate the response to elevated levels of CO_2. Evidence exists to suggest that cell-cell coupling via gap junctions, may be an important contributor to the HCVR, yet the extent to which this is relevant to 5-HT and its effects on the HCVR are unclear.

Anatomical and morphological evidence of gap junctions, as well as evidence of electrical coupling in CO_2 chemosensitive brainstem regions has suggested that gap-junction coupling may play a role in CO_2 chemosensation (40). Global gap junction uncoupling agents have been demonstrated to abolish the response to hypercapnia in the arterially perfused adult rat (unpublished

observation). Furthermore, previous work from our laboratory has demonstrated that mice deficient in the gap junction protein connexin-32 (Cx32) exhibit an altered hypercapnic ventilatory response (unpublished observation). Our laboratory has previously identified and characterized gap junctions in several chemosensitive brainstem regions, including the presence of Cx 32 in the raphe(174). The role of gap junctions, and particularly Cx 32, in the serotonergic modulation of the hypercapnic ventilatory response, however, is unknown.

It is generally accepted that 5-HT exerts a stimulatory effect on breathing, and that 5-HT release as a result of chemosensor activation, acts on downstream receptors to increase the frequency and depth of breathing. The selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLX) microdialyzed into the medullary raphe of conscious rats enhanced the HCVR (184). While no effects were noted with systemic FLX treatment, later studies from our lab and others have shown that in chronic, systemic FLX-treated mice, there is excitation of respiratory output during eupnea (6, 193). These observations are consistent with the role of 5-HT as an important excitatory neuromodulator of the HCVR.

We hypothesized that Cx32 –mediated gap junction coupling is necessary for maintaining excitatory serotonergic neuromodulation of the HCVR, and that release of 5-HT in response to elevated CO2 is impaired in mice deficient in Cx 32. Therefore, elevated levels of endogenous 5-HT, produced by chronic FLX

treatment, would lead to enhancement of the HCVR in wild-type mice and rescue the HCVR in mice deficient in Cx-32.

The current study aims to investigate whether the altered hypercapnic ventilatory response observed in Cx32-deficient mice is mediated, at least in part, by the serotonergic system. To evaluate this possibility, we examined the effects of chronic excess endogenous 5-HT produced by selective 5-HT reuptake inhibition on the hypercapnic ventilatory response in wild-type Cx32 KO mice.

METHODS

Implantation of Osmotic Pumps in Mice and Chronic Exposure Protocol:

For the chronic exposure portion of our experimental protocol, drug or vehicle will be delivered by miniosmotic pumps subcutaneously implanted in mice (n=62). Male and female Connexin 32 knockout (Cx32KO) mice were used alongside C57BL/6 mice (the genetic background of the knockout.)

Mice were anesthetized using isofluorane in a chamber, and maintained in a surgical plane through the use of a modified nose cone containing a gauze pad dipped in isofluorane. Pumps were implanted maintaining aseptic technique. The skin over the implantation site was cleaned and cleared of fur and a mid-scapular incision was made. A sterile hemostat was inserted into the incision for blunt dissection of a pocket just large enough to contain a filled miniosmotic pump

(Alzet, Model 1004). These pumps were previously filled according to manufacturer's instruction to contain either the selective serotonin reuptake inhibitor (SSRI) fluoxetine (0.02 mg/µl, dissolved in vehicle) or vehicle (50% DMSO, 50% saline. Wild-type males: n= 9 FLX, 8 VHCL; Wild-type females: n= 10 FLX, 8VHCL; Cx 32 KO males: n= 7 FLX, 6 VHCL; Cx 32 KO females: n= 8 FLX, 6 VHCL.)

After pump implantation, the incision was closed with wound clips or absorbable suture, and the mice given post operative analgesia (buprenorphine, 2 mg/kg, subcutaneously) as needed, and monitored for recovery of locomotor capabilities before being placed in a cage. Following pump implantation, all mice were maintained in a 12:12 hr light/dark cycle, between 20.7-21.8ºC, monitored and weighed daily, and allowed access to food and water ad libitum.

Our approach was to elevate endogenous serotonin through chronic administration of the SSRI fluoxetine using miniosmotic pumps implanted subcutaneously (see above). The mice were treated for 28 days, and the delivery rate for the pumps, containing either fluoxetine or vehicle was on average 0.11 µl/hr. For fluoxetine, this corresponds to ~2 mg/kg of drug released daily, (as estimated in a 25g mouse). The typical therapeutic dose in humans is roughly 0.5 mg/kg/day. However, clinical values vary widely, as determined by patient need. Thus, we have selected a dosage that is almost 4 times greater than the "average" clinical value, to reflect the higher end of therapeutic doses of fluoxetine (193).

Others utilizing the subcutaneous miniosmotic pump system have previously determined that steady-state of fluoxetine, and its active metabolite norfluoxetine, is reached within 48 hours of the start of infusion. Furthermore, at steady state, fluoxetine and norfluoxetine have been shown to be extensively distributed in the brain (75, 76). Therefore, we believe that this protocol allows us to reach and maintain steady-state levels of the drug for the majority of the duration of the chronic exposure, and that for the duration of this period the drug is active at the tissues of interest.

Surgical Preparation:

Following the 28-d fluoxetine exposure, the mice were surgically prepared for an acute experiment. All mice were anesthetized with an intraperitoneal injection of urethane (~1.5 g/kg). The adequacy of anesthesia was regularly verified by absence of a withdrawal reflex to a noxious interdigitary pinch and was supplemented as neccessary (~0.15 g/kg, ip). The mice were supplied with a gas mixture of 40% O_2, 60% N_2 from a nose cone. Diaphragm EMG activity was recorded using a thin twisted silver wire bipolar electrode inserted into the right side of the diaphragm (just right of the midline) while the mice breathe spontaneously. Body temperature was measured using a rectal probe and maintained at ~37°C throughout the experiment, using a heating pad and a heat lamp as necessary.

Experimental Protocol:

Stable baseline EMG activity was recorded for at least 10 minutes while the mouse breathed 60% N_2:40% O_2. The gas mixture was then changed to one containing 53% N_2:40% O_2:7% CO_2 (hypercapnic trial) for 10 minutes, after which the gas mixture was changed back to the baseline (control) mixture. At least 20 minutes was allowed for the mouse to recover, and then the gas mixture was switched to 100% N_2 (anoxia) to assess the HVR and gasping. Anoxia was maintained until terminal gasps were observed or all breathing efforts and heart rate ceased.

Data Acquisition and Analysis:

The EMG signal was amplified (1k), notch filtered at 60 Hz, and analog filtered between 10 Hz and 1 kHz. Filtered signals were rectified, and a moving average was obtained using a third-order Paynter filter with a 50-ms time constant. The raw and moving-averaged EMG activity was recorded on a computer at a sampling rate of 2 kHz (Chart 5.0, PowerLab, ADInstruments) and on digital tape at a sampling rate of 2.5 kHz (DAT, CygnusTechnologies) for off-line analyses (MatLab 7.0.1).

Individual traces of time series data from the PowerLab recordings were output for temporal analyses similar to as previously described (SA 1 and 2). However, for baseline EMG activity and the hypercapnic ventilatory response,

average values for these variables were calculated from 20 consecutive respiratory cycles during the last minute of each condition.

All data was reported as mean±SE. For data that are presented as paired data (*i.e.*, before and after design), statistical significance was assessed using one- and two-way analysis of variance (ANOVA) with repeated measures and the nonparametric Friedman's test, followed by *Holm-Sidak post hoc* analyses for multiple comparisons. For data that are presented in an unpaired design, statistical significance was evaluated using one- and two-way ANOVA and the nonparametric Friedman's test, followed by *Holm-Sidak post hoc* analyses for multiple comparisons. Comparisons of less than three groups were made using paired or unpaired Student's t-test, as appropriate. The criterion level for determination of statistical significance was set at $P<0.05$.

RESULTS

Chronic FLX Administration:

Throughout the duration of the chronic protocol, no adverse effects were noted among any of the treatment groups. All mice gained comparable amounts of weight for their sex (Fig V.1). Slight behavioral changes, consistent with previous literature, were noted in the FLX-treated mice, including reduced locomoter activity and increased amenability to handling (57).

Wild-type response to Hypercapnia:

In both VHCL-treated mice of both sexes and FLX-treated male mice CO_2 exposure produced an increase in minute activity, as expected. However, no significant change in minute activity was seen in FLX- treated female mice (Fig.V.2). Burst amplitude in all groups, while highly variable, increased in the majority of mice in all groups. However, while CO_2 produced significant increases in frequency in all VHCL-treated mice as well as in FLX-treated males, FLX-treated females displayed a significant fall in frequency under hypercapnia (Fig.V.2). FLX- treated females exhibited a somewhat elevated basal frequency as compared to other groups although this was not statistically significant (P=0.382). In all groups, CO_2 produced a shortening of T_I, however FLX- treated females displayed a marked lengthening of T_E, mediating a fall in frequency under hypercapnia (Fig.V.3).

Hypercapnic Ventilatory Response in Cx 32 deficient mice:

Cx-32 KO mice in all treatment groups exhibited no significant increase in minute activity under CO_2 (Fig.V.2). Female CX32KO mice appeared to show reductions in minute activity under CO_2, albeit insignificant (P=0.150). An increase in amplitude under CO_2 was evident in VHCL treated mice, but not in mice treated with FLX. While frequency and T_E remained unchanged under hypercapnia in all groups, only male Cx 32 KO mice receiving FLX displayed a shortening of T_I under CO_2 (Fig.V.3).

Changes in Respiratory Activity During Recovery from Hypercapnia

Increased respiratory drive, including that from a hypercapnic stimulus, is assosciated with increased incidence of augmented bursts or sighs (99). Augmented bursts, displaying typical characteristics of increased amplitude and slightly increased post-sigh interval (Fig. V.4), were observed in all experimental groups. The number of augmented bursts in each experiment varied from 1 to 9, and typically occurred towards the end of the hypercapnic trial and during the recovery period (data not shown).

In addition to typical augmented bursts, which occurred almost exclusively as single bursts (Fig.IV.4) we noted an additional atypical change in respiratory activity during the last 10-15 minutes of recovery (Fig. V.4). In this pattern of activity, diaphragm EMG bursting would spontaneously convert from a normal pattern and rhythm to an extended series of augmented-burst like activity, before reverting back to the normal output. The bursts during this pattern displayed markedly increased amplitude, T_I, and T_E, and consequently slower burst frequency, when compared to bursts taken from the period immediately preceding and following the change (Fig. V.5). The occurrence of these patterns was markedly more frequent in female mice of both genotypes treated with FLX (Table V.1).

Discussion

The data in this study show that 28-day treatment with fluoxetine failed to enhance the hypercapnic ventilatory response in wild-type male mice, while blunting or abolishing the response in females. In contrast to elevations in respiratory frequency exhibitied under hypercapnic conditions by VHCL treated mice of both sexes as well as FLX-treated males, FLX-treated females demonstrated significant reduction in frequency with hypercapnia. This fall in frequency was mediated primarily by an increase in T_E. However, T_I was significantly shortened with hypercapnia in all groups.

Male and female VHCL treated Cx-32KO mice showed blunted hypercapnic responses, consistent with previous findings. We observed no changes in minute activity with CO_2 in these mice, as well as in Cx-32KO mice on fluoxetine. The increase in burst amplitude that was noted in VHCL-treated mice was not evident in mice receiving FLX, suggesting that the deficit leading to the lack of HCVR in this genetic model is not supplied by FLX-treatment. However, male CX-32KO mice receiving fluoxetine were the only Cx-32 deficient group to display a reduction in T_I during hypercapnia, in a manner similar to that demonstrated in wild-type mice. Together these observations indicate that chronic elevation of endogenous 5-HT by FLX may impair chemosensitivity in female mice. However, FLX-treatment in male mice deficient

in Cx-32 may help to rescue certain features of the HCVR that are normally lost in the knockout mouse.

To our knowledge, this is the first attempt to characterize the HCVR in female rodents chronically exposed to systemic fluoxetine. While our data did not show evidence that FLX-treatment enhanced the HCVR, consistent with previous reports from male rats (184), we uncovered an interesting sexual dimorphism in the effects of FLX on the HCVR (see *Sex-specific effects of FLX*). It is to be noted that our data did not replicate the enhancement of eupneic breathing seen in previous work using male mice chronically exposed to higher doses of systemic FLX. Therefore it is unclear if and how higher doses of FLX may alter the HCVR.

This study is also the first to address the effects of fluoxetine on the HCVR in the Cx-32 deficient model, which had been previously shown to display an impaired response to increased levels of CO_2. It has been suggested that gap junctions may function to increase synchronization of the neuronal network in response to a hypercapnic stimulus. CO_2 chemosensation in rodents has been demonstrated to undergo developmental changes, with a critical period of very low chemosensitivity around day P8 (181). This may correlate to a critical period for (15, 201). Interestingly, Cx-32 expression is minimal before day P7, and by day 14 is at ~25% of adult expression levels (201). This, along with our previous

observations from the Cx-32 deficient mouse, suggests that Cx-32 may play a role in CO_2 chemosensation.

While we hypothesized that Cx-32 deficiency may lead to impaired release of 5-HT, and thusly impairment of the HCVR, our data do not suggest that chronic FLX treatment is entirely sufficient to restore the HCVR. However, our data do indicate that in Cx-32 KO males, some degree of rescue is possible with chronic, systemic FLX treatment. This suggests that, to some extent, the blunted chemosensitivity displayed by Cx-32 deficient males may be a function of insufficient 5-HT release. However, as the shortening of T_I was the only feature evident in the Cx-32 KO males consistent with the wild-type HCVR, the recovery of function is incomplete. Therefore the failure of the HCVR in the Cx 32 deficient mouse is most likely multifactorial. It is unclear if a higher dose of FLX would lead to increased recovery in male Cx-32 KO mice (see *Sex-Specific Effects of FLX*).

Sex-specific effects of fluoxetine

The sexual dimorphic changes in the HCVR in response to fluoxetine, both in wild-type and Cx 32 KO animals, could be attributable to a number of differences in male v. female physiology. FLX has been demonstrated to show sex-specific effects when chronically administered in mice. First, the pharmacokinetics of FLX differs in that female mice metabolize FLX faster and produce higher levels of the active metabolite norfluoxetine. FLX is among the

slowest metabolized of all antidepressant agents, and the half life of norfluoxetine is considerably longer than that of FLX(70). This may account for the increase in basal respiratory frequency seen in the FLX- treated wt females and in previous reports using male rodents treated with chronic FLX at higher doses. The failure of the wild-type female HCVR however, suggests that higher doses might not necessarily lead to improved HCVR, particularly as the level of 5-HT neurotransmission in females may be stimulated for additional reasons, including the level of sex hormones.

The exact mechanisms involved in the influence of sex hormones on central neural control of breathing are not well understood. Evidence suggests gonadal steroids may influence neural respiratory control, including chemosensation, in a number of ways (See Behan 2002, for a review). Estrogen and progesterone have been shown to increase 5-HT neurotransmission, and circulating levels of 5-HT have been shown to fluctuate with estrus cycle in rodents. Unfortunately it is not known if the primary effects of sex hormones occur at the level of peripheral or central chemosensors or the many respiratory related regions they project to including brainstem rhythm generator or premotor neurons, or the downstream respiratory motoneurons (15). While these differences appeared to not affect the HCVR in VHCL control mice, the marked difference in HCVR in FLX treated female mice suggests that sexual dimorphisms may interact with FLX-activity. For example, it is not known how the level and activity of the wide variety of 5-HT receptor subtypes is altered in

response to FLX treatment in male v. female mice. Interestingly, mice treated with FLX have shown sex specific differences in neural proliferation and production of BDNF in other regions of the brain (70). Both of these factors may alter control of breathing and CO_2 chemosensation.

Interestingly, it has also recently been shown that SSRIs also upregulate neurosteroids in a manner independent of their activity as SSRIs, and at lower doses than necessary for that function(147). Neurosteroids, including allopregnanalone, have been demonstrated to have profound effects on breathing (155), and it has been previously suggested that the effects of neurosteroid exposure may be sex-specific (56, 64, 207, 208). We have yet to uncover how and to what extent these multiple, complex factors may influence the interaction between 5-HT and the HCVR. The emergence of atypical changes in respiratory output during recovery from hypercapnia is almost certainly the result of the interaction of FLX with female-sex specific factors, as these patterns were notably enriched in female mice receiving FLX, regardless of genotype. The significance of the emergent pattern, as a compensatory mechanism, deleterious effect, or innocuous feature of breathing, also remains unclear.

Nonetheless, the evidence of a sexually dimorphic respiratory phenotype is particularly interesting in light of the sexual dimorphism evident in cases of SIDS. Male infants are more likely to die from SIDS as compared to female infants. While the failure to respond to CO_2, postulated to be involved in the

etiology of SIDS, evidenced in our studies appears to be in the female mice, it should also be noted that the extracellular levels of 5-HT found in infants who have died from SIDS was recently found to be lower than that of controls(49), and not chronically elevated (as in this current work). Therefore the value of our finding is not that it replicates the condition seen in SIDS, but that it suggests a physiological link between sex, extracellular 5-HT, and a pathology of breathing. Further investigations into the nature of this relationship will hopefully help us to build better models of SIDS.

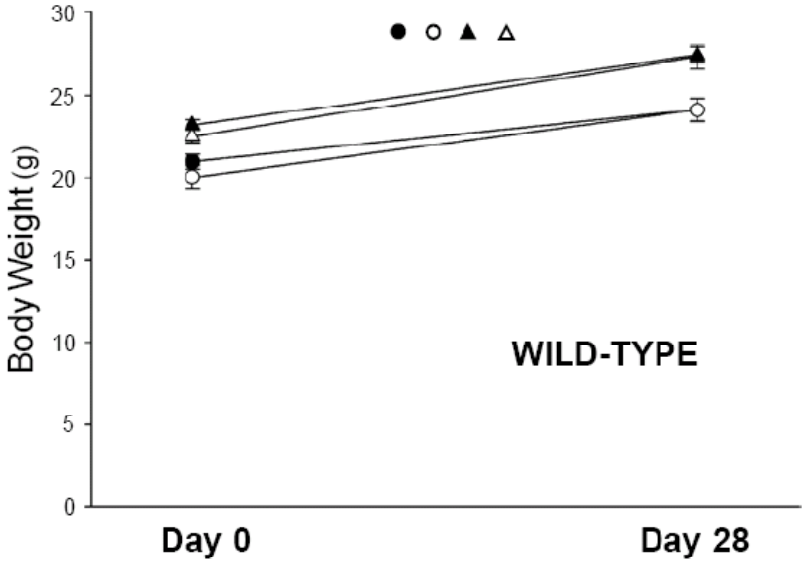
Limitations and Future Directions

Indeed, a limiting factor of most studies to date, including this one has been the failure to account for estrus cycle. We plan to further investigate the interaction between sex hormones and 5-HT mediated effects on HCVR, using gonadectomized mice.

An important limitation of the *in vivo* diaphragm EMG preparation is the requirement for anesthesia. While urethane is commonly employed for its minimal effects on respiratory output, there is a growing body of evidence to suggest that urethane may alter ventilatory responses to some extent. Furthermore, it has been suggested that the relative importance of the various chemoreceptor sites is state-dependent. It is not known how active the medullary 5-HT neurons are under anesthesia. Therefore, further studies will require the employment of additional experimental preparations, such as the arterially-perfused *in situ* mouse, which is a decerebrate preparation.

Through ongoing work analyzing tissue obtained from mice used in these studies, we hope to gain insight into how chronic FLX treatment is altering the levels of several serotonergic receptors and markers. By continuing to apply these approaches in gonadectomized rodents, we hope to clarify how changes in serotonin-related protein expression may be influenced by sex-hormones. Through these studies, we also hope to clarify if the Cx-32 KO respiratory phenotype is related to an underlying 5-HT abnormality. This information will help to guide future studies, and hopefully will further clarify the mechanisms involved in this complex but critical response.

Figure V.1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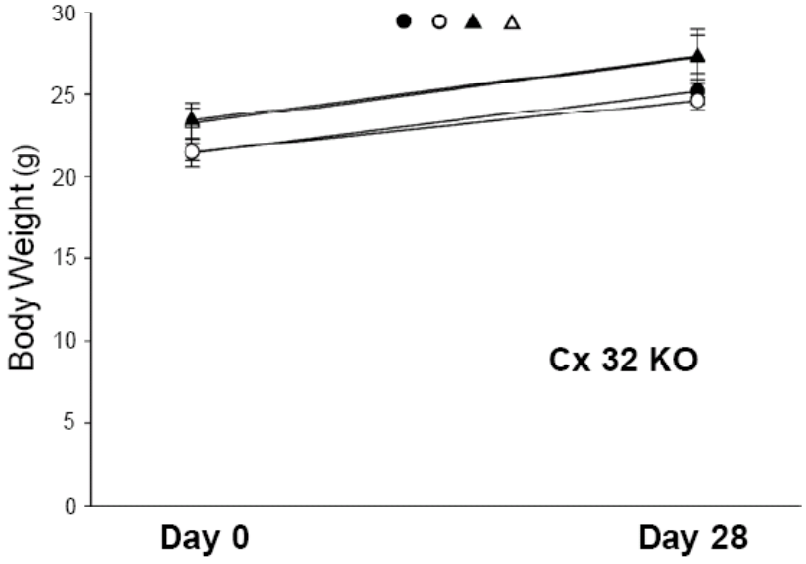
25
20
15
10
5
0
Body Weight (g)
WILD-TYPE
Day 0
Day 28


30
25
20
15
10
5
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Body Weight (g)
Cx 32 KO
Day 0
Day 28

Figure V.2

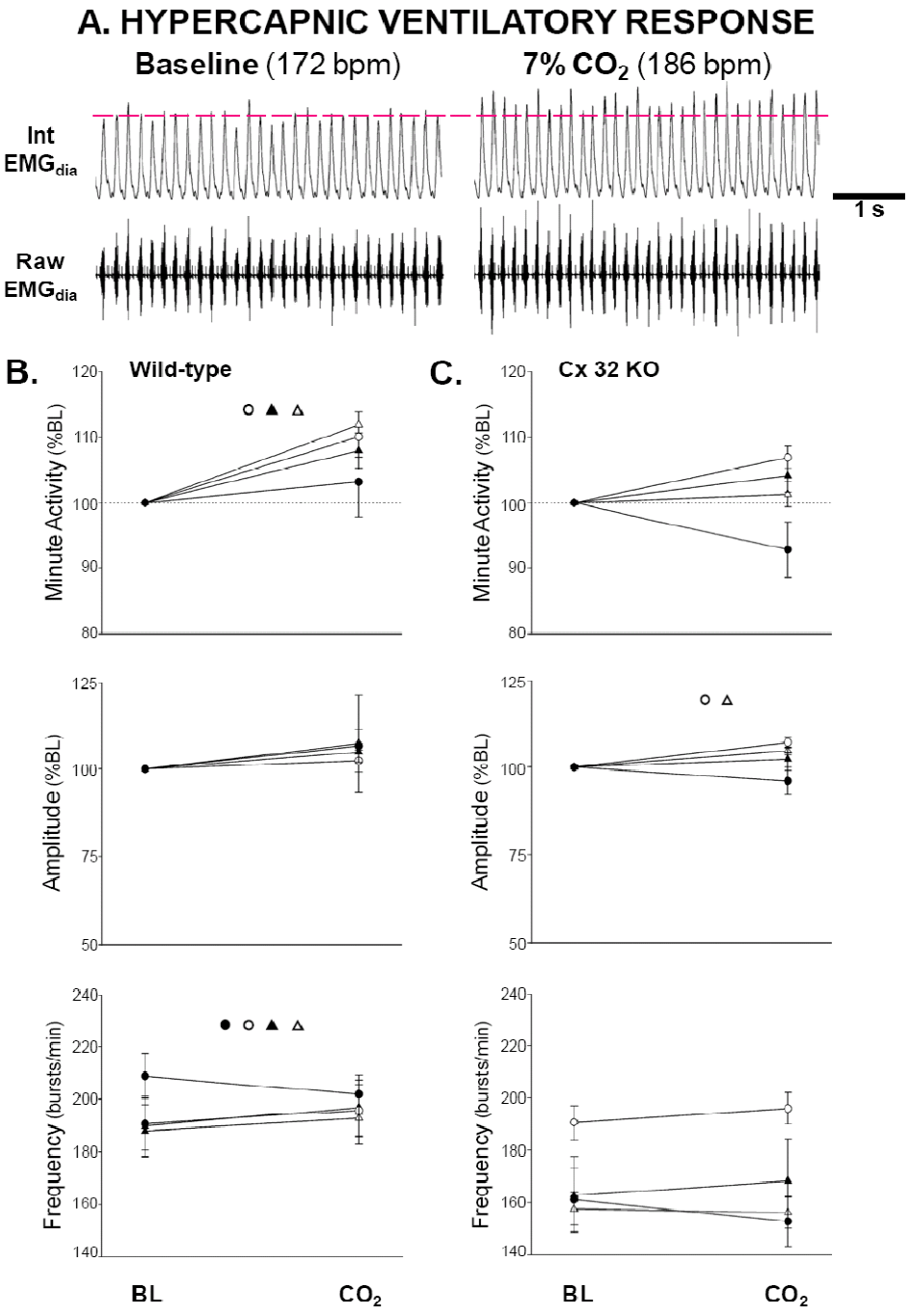
A. HYPERCAPNIC VENTILATORY RESPONSE
Baseline (172 bpm)
7% CO2 (186 bpm)
Int EMGdia
Raw EMGdia
1 s
B.
Wild-type
C.
Cx 32 KO
Minute Activity (%BL)
Amplitude (%BL)
Frequency (bursts/min)
BL
CO2

Figure V.3

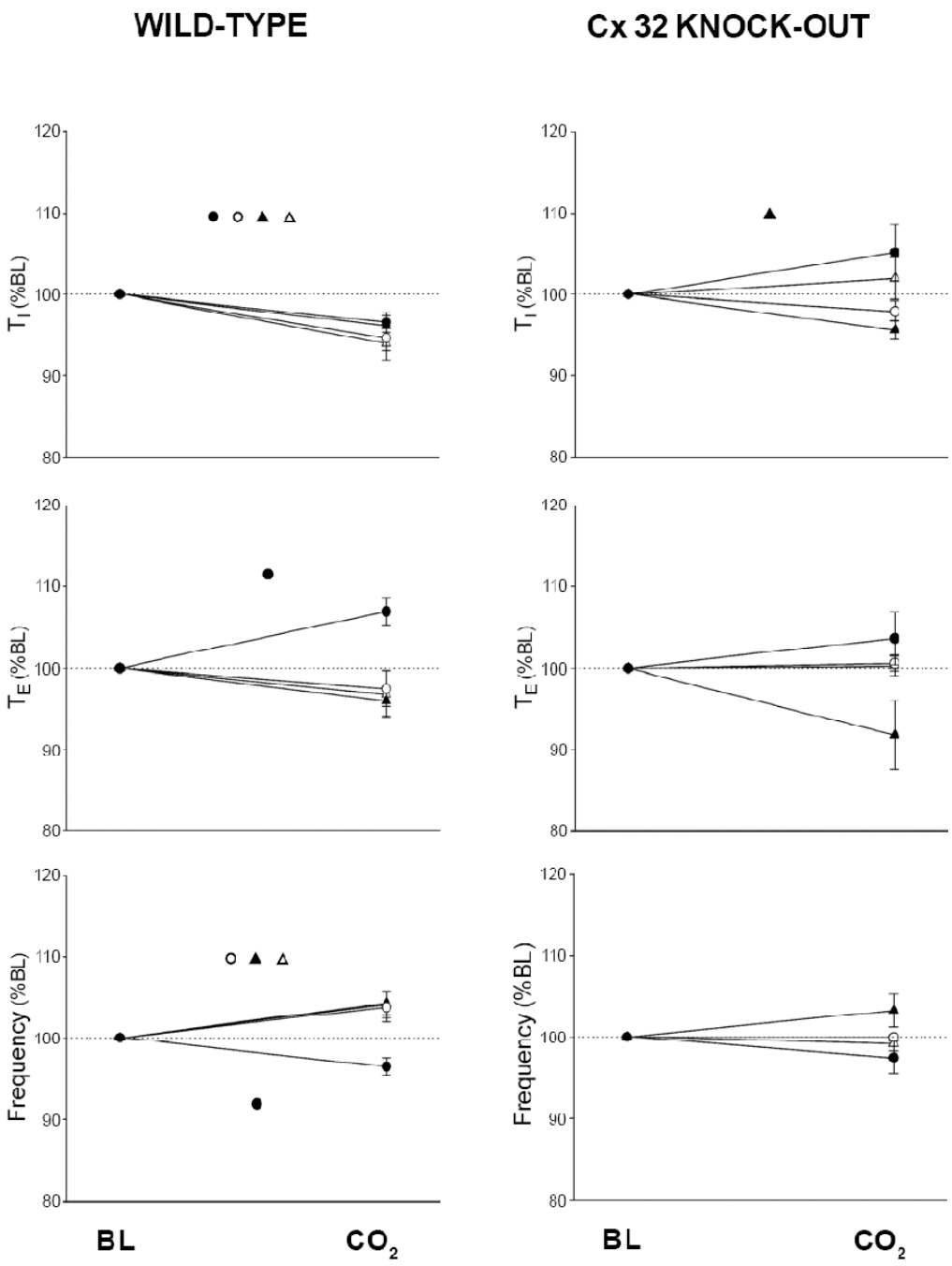
WILD-TYPE
Cx 32 KNOCK-OUT
120
110
100
90
80
T_I (%BL)
T_E (%BL)
Frequency (%BL)
Frequency (%BL)
BL
CO_2
BL
CO_2

Figure V.4

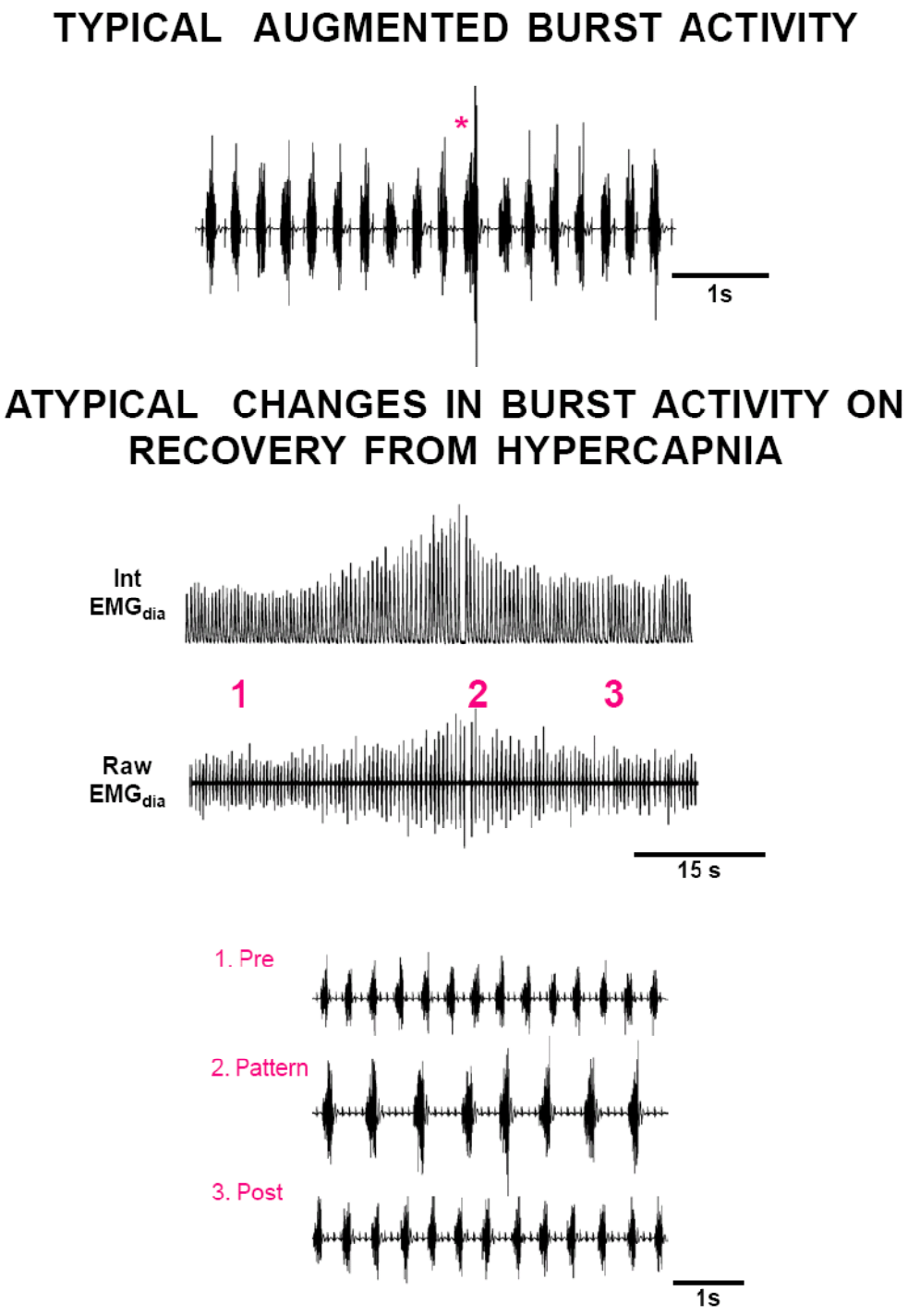
TYPICAL AUGMENTED BURST ACTIVITY
*
1s
ATYPICAL CHANGES IN BURST ACTIVITY ON RECOVERY FROM HYPERCAPNIA
Int EMG$_{dia}$
1
2
3
Raw EMG$_{dia}$
15 s
1. Pre
2. Pattern
3. Post
1s

Figure V.5

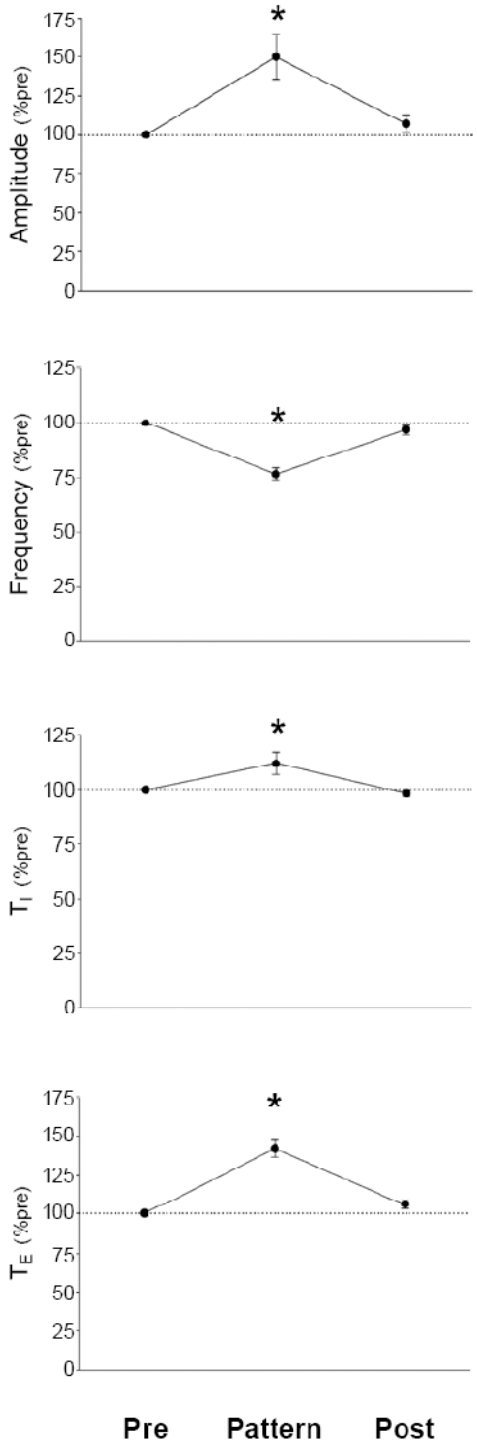
Amplitude (%pre)
Frequency (%pre)
T_I (%pre)
T_E (%pre)
175
150
125
100
75
50
25
0
*
Pre
Pattern
Post

Table V.1

VHCL	WILD-TYPE	Cx 32 KO
MALE	1/8	0/6
FEMALE	1/8	0/6

FLX	WILD-TYPE	Cx 32 KO
MALE	0/9	1/7
FEMALE	**7/10**	**6/8**

FIGURE LEGENDS

Figure V.1. Changes in weight over 28-day chronic treatment. All experimental groups, of both wt and Cx 32 KO mice, showed significant increases in weight over the duration of the chronic exposure. Circles represent female mice, triangles represent male mice. Closed symbols represent treatment with FLX, open symbols represent VHCL-controls. Symbols (○,●,▲,Δ) indicate statistically significant change ($P<0.05$)

Figure V.2. Changes in the hypercapnic ventilatory response after chronic exposure to FLX. **A.** Examples of raw and integrated diaphragm EMG taken before and during a 7% CO_2 challenge, taken from a VHCL control female. Hypercapnia produces an increase in burst frequency and amplitude. Effects of CO2 on minute activity, burst amplitude, and frequency in wt (**B)** and Cx 32 KO mice(**C**). Circles represent female mice, triangles represent male mice. Closed symbols represent treatment with FLX, open symbols represent VHCL-controls. Symbols (○,●,▲,Δ) indicate statistically significant change ($P<0.05$)

Figure V.3. Temporal changes under CO_2 after chronic exposure to FLX. Changes in burst duration (TI), time between bursts (TE), and frequency in wt and Cx 32 KO mice.Circles represent female mice, triangles represent male mice. Filled symbols represent treatment with FLX, open symbols represent VHCL-controls. Symbols (○,●,▲,Δ) indicate statistically significant change ($P<0.05$)

Figure V.4. Changes in respiratory patterning during recovery from hypercapnia. (Top) Example of a typical raw diaphragm EMG trace containing an augmented burst (preceded by asterix, *). (Bottom) Example of previously undescribed changes in respiratory activity and patterning, with expanded raw trace segments taken before (pre), during (pattern), and after (post) the change.

Figure V.5. Changes in amplitude and temporal characteristics before (pre), during (pattern), and after (post) atypical changes in respiratory activity and patterning during recovery from hypercapnia (normalized to pre).Asterix (*) indicate statistically significant change ($P<0.05$)

Table V.1. Proportions of each experimental group showing evidence of atypical changes in respiratory activity and pattern during recovery from hypercpania. Note that the incidence of the change is considerably higher in female mice receiving FLX in both wt and Cx 32 KO groups.

CHAPTER VI

Summary and Conclusions

The work presented in this dissertation was designed to (1) provide insight into the role of 5-HT acting on the 5-HT_{2A} receptor in central respiratory control, and (2) clarify the role of Cx 32 in serotonergic modulation of the hypercapnic ventilatory response.

5-HT neurons of the medullary raphe are commonly believed to function as important central chemosensors, firing and releasing 5-HT in response to elevated CO_2. 5-HT acting on downstream receptors was presumed to mediate the corresponding respiratory excitation under hypercapnic conditions. We hypothesized the identity of the pivotal 5-HT receptor subtype involved to be the 5-HT_{2A} R subtype, due to the excitatory effects on breathing elicited by 5-HT_{2A} R agonists, and the importance of endogenous 5-HT_{2A}R activation in the hypoxic ventilatory response and gasping. While our data suggest that some degree of 5-HT_{2A} R involvement may play a role in the normal HCVR, in the absence of 5-HT_{2A} R activation, a largely normal response to hypercapnia may still be elicited. Given the redundancy of chemosensors, this may not be a surprising result. However, our findings do illustrate that 5-HT_{2A} activation is not critical to HCVR formation. Future studies are necessary to yield insight into the interplay between the many chemosensitive sites, and their state-dependent importance.

The literature regarding 5-HT and control of breathing is fraught with contradictory observations. This may be attributed in some degree to the multitude of 5-HTR subtypes and the variety of experimental preparations utilized to study breathing. A portion of this dissertation (*see Ch.IV and Appendix*) has attempted to determine the contribution of $[K^+]_o$ to 5-HT_{2A} R blockade influenced ventilatory responses. While we found that the level of $[K^+]_o$ exerted only minimal effects, this type of systematic approach should be employed in future studies and applied to other potential preparation differences that may account for the inconsistencies in previous work. These studies will help in understanding how 5-HT influences ventilatory responses via its function as part of an integrated neuronal network.

This dissertation contains the first attempt to address the mechanism underlying the aberrant HCVR seen in Cx 32 KO mice. Previously, we knew of the expression of gap junction proteins, particularly connexin-32, in the medullary 5-HT neurons. Physiolgical evidence of impaired HCVR in Cx 32 deficient mice also existed. While we hypothesized that Cx-32 mediated cell-cell coupling was involved in the release of 5-HT in response to elevated CO_2, our findings do not strongly support this idea. Further work is being undertaken to determine if our chronic FLX treatment is raising levels of endogenous 5-HT, as well as what changes in receptor subtype expression are occurring over the duration of the chronic exposure. It is possible that irrespective of the basal level of 5-HT, the 5-HT neurons' ability to respond acutely to hypercapnia is still impaired in the Cx-

32 deficient model. However, if this is the result of impaired 5-HT release, or the failure to integrate or synchronize with other chemosensitive sites, remains to be determined.

Another complication in studying the effect of 5-HT on breathing is that 5-HT is known to exert widespread systemic effects. This is evident when considering the extent of confounding factors that may be influencing our findings in the experiments involving chronic systemic fluoxetine administration. Again, a systematic approach is required to determine if and what other factors including hormones may be interacting with the 5-HT system to influence the HCVR and recovery from hypercapnia in female mice. The respiratory effects of SSRIs are not well understood, and while a large proportion of human patients who have been prescribed these drugs are female, past studies of respiratory effects of SSRIs have focused only on male rodents. The work presented in this dissertation represents the first attempt to characterize the effects of SSRIs on breathing in female rodents. That our findings suggest that chronic FLX treatment may impair the HCVR, further highlights the importance of this complicated question. Sexual dimorphisms in chronic-FLX induced changes in the HCVR that were uncovered in this work represent another important finding, as several known pathologies of breathing (including SIDS) have been shown to occur more frequently in one sex over the other.

Finally, the research presented in this dissertation does bear particular relevance for understanding SIDS. The eminent hypothesis of SIDS etiology proposes that an underlying abnormality of the 5-HT system predisposes an infant to fail to respond appropriately to environmental stressors, including hypoxia and hypercapnia. The work presented in this dissertation has shown that the 5-HT_{2A} is involved in the normal hypercapnic ventilatory response, and plays a critical role in the response to hypoxia. Our findings do not support the likelihood that a pharmaceutic with FLX-like properties might be able to correct or enhance the HCVR. Furthermore, we suggest that sustained alterations in the levels of endogenous 5-HT may impair the ability to respond to elevations in CO_2. This may be relevant to understanding how 5-HT abnormalities in the SIDS originate. Furthermore, our findings indicate that certain pharmacological strategies, designed to correct aberrant ventilatory responses, may in fact produce counterproductive results.

In 1992 Jacobs and Azmitia observed, "…although serotonin has been implicated in a wide variety of physiological processes, it appears to be essential to none of them." At the time, this statement suggested that 5-HT exerts widespread but mostly modulatory, rather than critical, effects. However, what we have since learned from the victims of SIDS suggests that the consequences of irregularities within the 5-HT system could be catastrophic. Over the years, it is becoming increasingly apparent that 5-HT may play a more crucial role in respiratory related behaviors than previously thought. The importance of 5-HT in

fundamental survival mechanisms, has never been so apparent as when considering the role of 5-HT_{2A} activation in the response to severe hypoxia, as demonstrated in previous work and that presented here. While considering the role of 5-HT in responses to elevated CO_2, it is easy to fall back on a "implicated, but not essential" type of explanation. However, much work remains to be done. Indeed, as we make strides in clarifying the importance of 5-HT, we are concurrently discovering how interdependent and complex the system as a whole truly is. The goal for the future, albeit a difficult one, must be to embrace the difficult questions and increase our understanding of these many intricacies of 5-HT neuromodulation, if we are to ever understand where, how, and when 5-HT becomes critical to survival,

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APPENDIX

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Influence of 5-HT_{2A} receptor blockade on phrenic nerve discharge at three levels of extracellular K^+ in arterially-perfused adult rat

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Abstract.
Recent observations from *in vitro* rodent preparations suggest an important role for the serotonin-2A (5-HT_{2A}) receptor in eupneic (basal) and gasping respiratory activities, although the precise role appears to be different amongst preparations. Since these *in vitro* preparations are typically supplied with artificial cerebrospinal fluid (aCSF) containing elevated (and different) levels of K^+ to increase neuronal excitability, the role of endogenous activation of 5-HT_{2A} receptors in these respiratory behaviors under "normal" levels of extracellular K^+ ($[K^+]_o$) requires clarification. This investigation sought to evaluate the influence of $[K^+]_o$ on the 5-HT_{2A} receptor-mediated effects onbasal respiratory activity and the phases of the hypoxic ventilatory response (HVR), including ischemia-induced gasping using an arterially-perfused adult rat preparation. Our data demonstrate that at each level of $[K^+]_o$ examined, 5-HT_{2A} receptor blockade increases basal phrenic burst frequency, decreases amplitude, alters burst pattern, and eliminates the phases of the HVR, supporting an important role for 5-HT_{2A} receptors in respiratory control, and indicating its independence on $[K^+]_o$.

1 Introduction

Over the years, studies have identified important modulatory roles for serotonin (5-HT) in various aspects of central respiratory control. Recent studies have aimed to delineate the roles of the various 5-HT receptor subtypes in respiratory-related behaviors. Amongst these studies, recent observations from *in vitro* rodent preparations have demonstrated that activation of the excitatory 5-HT_{2A} receptor subtype increases basal respiratory burst frequency (Pena and Ramirez 2002; St. John and Leiter 2008; Bale, Warren, and Solomon 2008), but the effects of receptor blockade are somewhat variable. In the neonatal mouse transverse medullary slice preparation, for example, bath application of the 5-HT_{2A} receptor antagonist ketanserin (KTN) depresses or abolishes, respiratory bursts (Pena and Ramirez 2002) while in the arterially-perfused juvenile/adult rat preparation, blockade of 5-HT_{2A} receptors increases basal respiratory burst frequency (St. John and Leiter 2008). Similarly, in the mouse slice preparation, 5-HT_{2A} receptor blockade abolishes anoxic gasping (Tryba, Pena, and Ramirez 2006) while during blockade in the arterially-perfused rat, ischemic gasps persist, though modified (Toppin, Harris, Kober, Leiter, and St. John 2007).

These observations indicate that endogenous 5-HT acting on 5-HT_{2A} receptors may play an important role in basal (eupneic) and gasping respiratory activities, yet this role is not identical among different *in vitro* rodent preparations. Several differences between these studies and preparations exist, including species and age of the preparation, amount of neural substrate, artificial cerebrospinal fluid (aCSF) and drug delivery method, conditions used to elicit gasping, and level of intrinsic neuronal excitability (which is set, in part, by the level of K^+ in the aCSF); thus, additional studies are needed to identify the precise reasons for the observed differences. Here, we have begun to address the influence extracellular K^+ ($[K^+]_o$) on the role of endogenous 5-HT acting on the 5-HT_{2A} receptor in basal respiratory rhythm and the hypoxic ventilatory response (HVR), including ischemia-induced gasping.

2 Experimental Protocol

All experiments were performed in an arterially-perfused decerebrate adult rat preparation under protocols approved by the Institutional Animal Care and Use Committee at Stony Brook University in accordance with the NIH Policy of Humane Care and Use of Laboratory Animals. The general methods have been previously described in detail, and will not be repeated here (Solomon, Rodriguez, and Chon 2003).

We examined phrenic nerve discharge under baseline (eupneic) conditions and in response to an ischemic challenge before and during perfusion with the 5-HT_{2A} receptor antagonist ketanserin (KTN; 40 μM) in arterially-perfused adult rat preparation. Experiments were done at three levels of $[K^+]_o$: 3.0 mM K^+, similar to "normal" physiological levels (n=8); 4.25 mM K^+, as occasionally used in this preparation (n=8); and 6.25 mM K^+, the typical level of $[K^+]_o$ used in this preparation (n=8). Ischemic challenges were conducted to evaluate the phases of the HVR and gasping. For all experiments, baseline activity was recorded for a minimum of 10 min, before a bypass in the perfusion circuit was opened to redirect the perfusate away from the preparation (*i.e.*, ischemia) for at least 90 s, and then closed to re-establish perfusion and begin recovery (10 min). KTN was then added to the perfusate and 10 min allowed for drug effect, before reinitiating ischemia.

For each level of $[K^+]_o$, time series traces of phrenic nerve activity during basal and ischemic conditions were extracted from the experimental record for control conditions and perfusion with KTN. Inspiratory burst duration (T_I), the duration between bursts (T_E), time-to-peak (T_{peak}/T_I), burst amplitude, and frequency under each condition were determined. The phases of the HVR and the total number of ischemic bursts were also evaluated. All data are reported as mean±SE, with burst data normalized to baseline (control) conditions. Statistical comparisons were made using either a one-way ANOVA or a one-way ANOVA with repeated measures, as appropriate, followed by Holm-Sidak *post hoc* analyses.

3 Results and Discussion

3.1 Effects of KTN on Basal Phrenic Nerve Discharge

At each level of $[K^+]_o$, perfusion with KTN decreased phrenic burst amplitude, increased frequency, and altered burst shape from augmenting to bell-shaped (Fig. 1). Frequency changes were mediated by significant reductions in both T_I and T_E, and changes in burst shape were reflected by T_{peak} shifting to an earlier time point (by ~15-20%) in the burst. The magnitude of the amplitude, frequency, T_{peak}/T_I, and T_E changes were independent of the level of $[K^+]_o$ (P=0.25-0.90) while those in T_I were dependent upon $[K^+]_o$ (P=0.03 for 3.0 mM and 4.25 mM vs. 6.25 mM).

These observations suggest that endogenous activation of 5-HT_{2A} receptors participates in establishing the general timing and patterning characteristics of basal phrenic nerve discharge, and that this role is not largely dependent upon the level of $[K^+]_o$-mediated respiratory network excitability. Previously in this preparation (aCSF $[K^+]_o$= 6.25 mM), KTN (5-30 μM) was reported to increase basal inspiratory burst frequency and reduce amplitude, but was not statistically significant (Toppin et al. 2007). Our findings support this observation and further indicate that KTN alters burst pattern, suggesting a role for 5-HT_{2A} receptors in shaping the inspiratory burst.

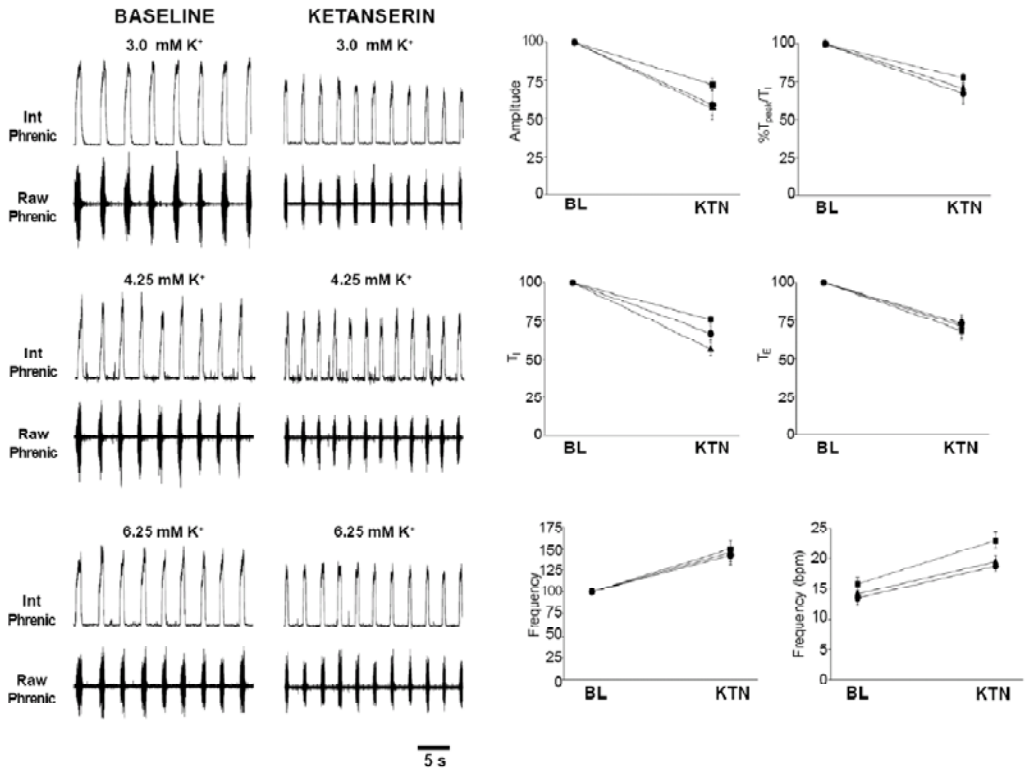
3.2 Effects of KTN on the HVR and Ischemia-induced Gasping

At each level of $[K^+]_o$ under control conditions, the HVR was characterized by three phases: (1) respiratory excitation (peripheral chemoreflex, PCR) with enhanced phrenic burst amplitude and frequency, and continued augmenting burst discharge pattern. (2) a period of transition bursts and/or apnea, and (3) gasping, characterized by decrementing discharge pattern and markedly prolonged duration between bursts (Fig. 2). Perfusion with KTN disrupted the typical phases of the HVR; the transition/apneic period disappeared and the PCR was followed immediately by a series of ischemic bursts exhibiting a decrementing discharge pattern, but were not accompanied by a marked prolongation of T_E (Fig. 2). The response was not influenced by the level of $[K^+]_o$, and under both control conditions and perfusion with KTN, ischemia elicited a similar number of phrenic bursts (from the onset of the PCR).

These observations indicate that endogenous activation of 5-HT_{2A} receptors participates in establishing the phases of the HVR, but its role in gasping is less clear. Our data demonstrate that ischemia induces phrenic bursts that share burst timing and patterning characteristics with those of hypoxia/ischemia-induced gasps, yet are not separated by long expiratory pauses (T_E), occurring over shortened timeframe. Additional studies are needed to clarify whether these bursts represent primary gasps or some other altered pattern of hypoxic inspiratory motor output.

4 Conclusions

To our knowledge, this study represents the first attempt to evaluate the contribution of $[K^+]_o$, including physiologically relevant $[K^+]_o$ levels, on the role of endogenous 5-HT_{2A} receptor activation in central respiratory control *in vitro*. Our data support a role for endogenous activation of 5-HT_{2A} receptors in the expression of basal phrenic nerve discharge and the HVR, including ischemia-induced gasping, and further suggest that this role independent of the level of $[K^+]_o$-mediated respiratory network excitability, suggesting inconsistencies in the literature regarding the effects of 5-HT_{2A} receptor blockade on eupnea and gasping are not due to differences in $[K^+]_o$-mediated respiratory network excitability.


BASELINE
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Int Phrenic
Raw Phrenic
4.25 mM K+
4.25 mM K+
Int Phrenic
Raw Phrenic
6.25 mM K+
6.25 mM K+
Int Phrenic
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5 s
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%Tpeak/TI
TI
TE
Frequency
Frequency (bpm)
BL
KTN

Figure 1

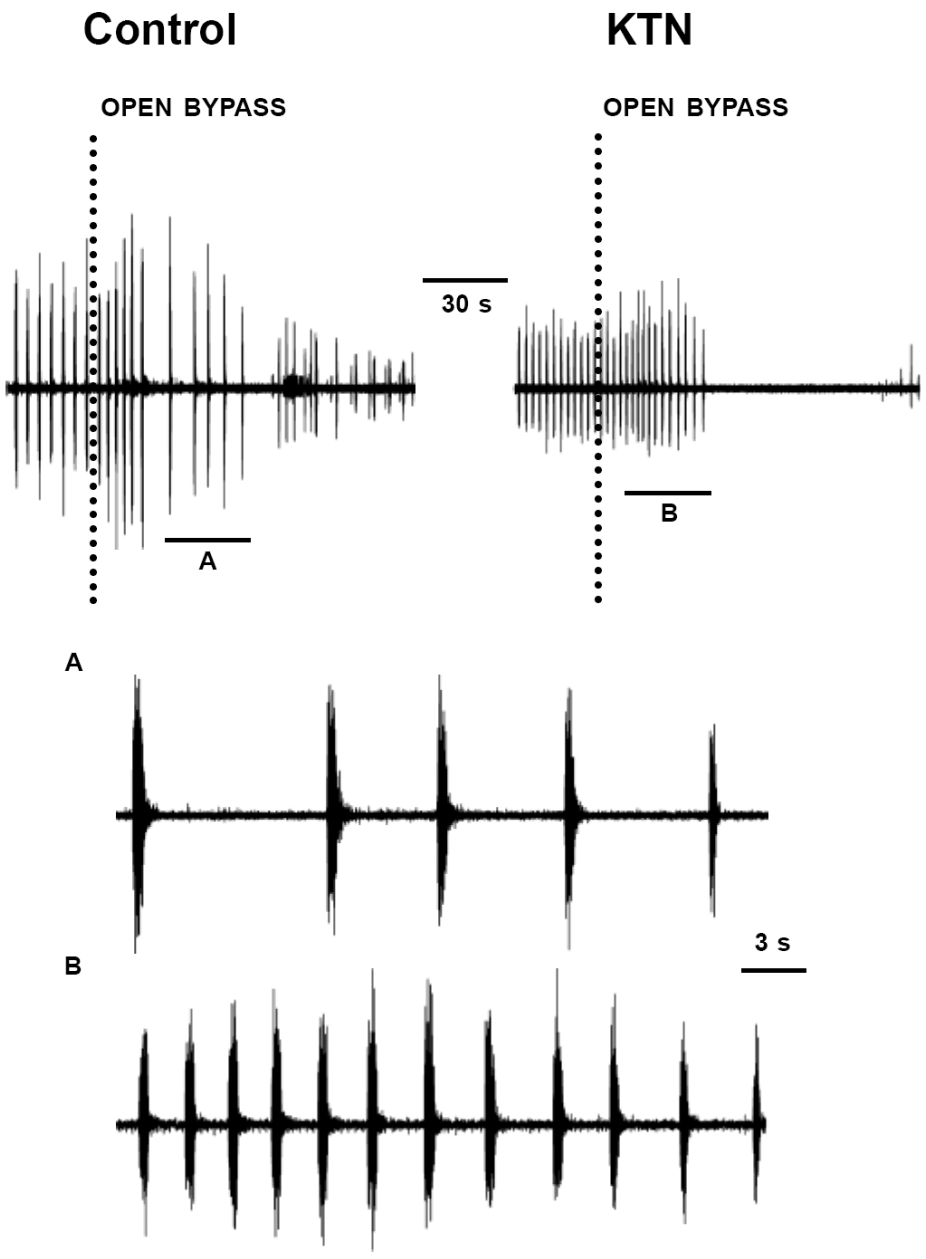

Figure 2

Figure Legends

Figure 1. Example of traces of basal phrenic nerve dischare under baseline conditions and during perfusion with KTN at 3 levels of $[K^+]_o$. Summary data show effects of KTN on patterning (amplitude, T_{PEAK}/T_I) and timing (TI, TE, frequency) of phrenic nerve discharge at 3.0 mM (circle), 4.25 mM (square) and 6.25 mM (triangle) K^+. *, statistically significant difference between baseline (BL) and KTN for each $[K^+]_o$ examined; bracket statistically significant difference (P= 0.025) between pairs identified.

Figure 2. Example traces of phrenic nerve discharge showing the response to ischemia under control conditions and during perfusion with KTN at 4.25 mM $[K^+]_o$. Top panel shows entire ischemic challenge and (A) and (B) show expanded trace at time points indicated by bars.

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