THE CONTROL OF BREATHING IN THE GOLDEN-MANTLED GROUND SQUIRREL (Spermophilus lateralis)

by

MICHAEL BRUCE HARRIS

B.Sc. (Hons. Zoology), The University of British Columbia, 1990
M.Sc. (Zoology), The University of British Columbia, 1992

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Department of Zoology

The University of British Columbia
Vancouver, Canada

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ABSTRACT

The Golden-mantled ground squirrel (*Spermophilis lateralis*) breaths continuously during euthermia and episodically during hibernation. How and why this conversion occurs is unknown. Breathing is continuously shaped into a precise pattern which appropriately matches ventilation to metabolic demands. In all mammals, sensory inputs from pulmonary mechanoreceptors (carried in the vagus nerve), and a specific cluster of neurons located in the pons (the pneumotaxic center, PC) play key roles in modulating this pattern. The present investigation was designed to determine how the influence of these two inputs changes as squirrels enter hibernation, and if changes in the integration of these inputs could be responsible for producing the episodic breathing pattern observed during hibernation.

Ventilation in euthermic ground squirrels was critically dependent on intact vagus nerves. These animals did not breathe in the absence of vagal feedback. In anesthetized animals, on the other hand, ventilation continued post-vagotomy but the shape of individual breaths was altered. This suggests there is a powerful inhibition of breathing that is normally offset by vagal feedback, but which is removed by anesthesia. In hibernating animals, vagal feedback was even less critical, it increased the overall level of ventilation by increasing the length of breathing episodes.

Glutamatergic processes utilizing NMDA type receptors were shown to be involved in the expression of sleep and sleep-like states of central activation. They were also involved in producing the ventilatory response to hypoxia in anaesthetized and unanaesthetized animals. Finally, they also depressed breathing frequency during sleep, anesthesia and hibernation. All of these effects are deduced to arise from glutamatergic processes outside the PC, however.

Glutamatergic processes utilizing NMDA type receptors within the PC are deduced to

assist in the termination of inspiration in anaesthetized animals in a similar fashion to vagal feedback as has been shown in other mammals.

While removal of either vagal feedback or NMDA receptor-mediated processes in the PC had only modest effects on breathing pattern, removal of both produced an extreme prolongation of inspiration (apneusis) in euthermic squirrels. In hibernating animals, removal of both inputs converted episodic breathing into a pattern of evenly spaced breaths. This latter observation suggests that integration of vagal feedback with glutamatergic processes (perhaps within the PC) is responsible for clustering breaths into episodes during hibernation. How the function of these inputs is transformed from one of shaping individual breaths to one of shaping episodes of breaths remains unknown.

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LIST OF ABBREVIATIONS

AW ..... Active wakefulness

Br/Ep ..... Breaths per episode

CPG ..... Central pattern generator

CRG ..... Central rhythm generator

CNS ..... Central nervous system

CSF ..... Cerebrospinal fluid

DRG ..... Dorsal respiratory group

ECG ..... Electrocardiogram

EEG ..... Electroencephalogram

EMG ..... Electromyogram

f_R ..... Respiratory frequency

$f_{R_{inst}}$ ..... Instantaneous respiratory frequency

KF ..... Kölliker-Fuse nucleus

MG ..... Morris Garages

MK-801 ..... (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801)

NMDA ..... N-methyl-D-aspartate

NPBL ..... Nucleus parabrachialis lateralis

NPBM ..... Nucleus parabrachialis medialis

NTS ..... Nucleus tractus solitarius

PBC ..... Parabrachial complex

PSR ..... Pulmonary stretch receptors

QW. ..... Quiet wakefulness

RTN ..... Retrotrapezoid nucleus

SWS ..... Slow-wave sleep

T_E ..... Expiratory duration

T_{EA} ..... Duration of expiratory flow

T_{EP} ..... Duration of the end-expiratory pause

T_I ..... Inspiratory duration

T_{IA} ..... Duration of inspiratory flow

T_{IP} ..... Duration of the end-inspiratory pause

T_{TOT} ..... Total respiratory cycle duration

$\dot{V}_E$ ..... Minute ventilation

V_T ..... Tidal volume

VRG ..... Ventral respiratory group

VX ..... Vagotomy or vagal blockade

Chapter 1

General Introduction

1.General Introduction

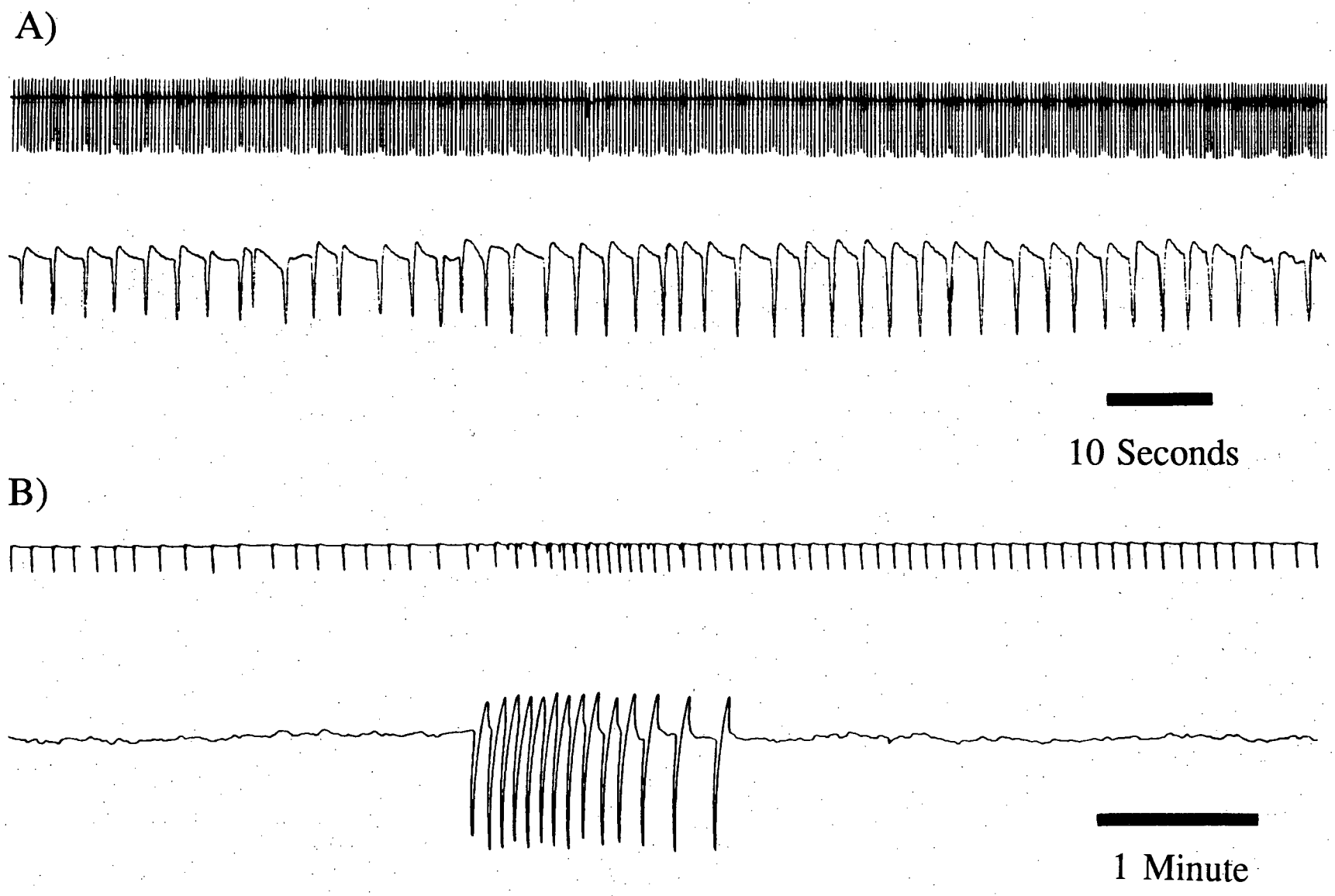
1.1 Preface

Many species of mammal employ deep torpor or seasonal hibernation as a strategy to overcome periods of resource limitation or environmental stress. All seasonal hibernators, however, maintain a core body temperature which is greater than ambient, and periodically arouse from hibernation to return briefly to euthermic (non-hibernating) body temperatures throughout the hibernation season (see Lyman, 1982 for review). The factors which underlie the regulation of homeostasis during hibernation have been the subject of many investigations (see Kilduff et al. 1993; Lyman, 1982 for review) but, for the most part, remain poorly understood. It is apparent, however, that physiological processes are actively regulated during hibernation.

The Golden-mantled ground squirrel (*Spermophilus lateralis*) is a sciurid rodent native to mountainous regions of western North America. These animals exhibit seasonal hibernation, during which their metabolic rate is less than 5 % of that during euthermia (Malan, 1982). Associated with this decline in metabolism, is a decline in ventilatory demand. Thus, during entrance to hibernation, both metabolic rate and breathing slow. Rather than simply slowing, however, breathing initially waxes and wanes and then gives way to a series of continuous breaths that are clustered into episodes and separated by prolonged periods of apnea. During established hibernation, breathing occurs in a stable episodic pattern (Fig. 1.1). This is regulated to meet ventilatory demands and remains responsive to ventilatory stimuli. The factors which convert from continuous breathing to breathing in episodes, and those which modulate the breathing within the episodes are, however, unknown.

Figure 1.1

Electrocardiogram (top tracing) and breathing traces (bottom tracing) obtained from (A) a euthermic ground squirrel, and (B) a hibernating ground squirrel with a body temperature of 7° C. Breathing records are respiratory impedance tracings and inspiration is a downward deflection.


A)
10 Seconds
B)
1 Minute

1.2 Influences of ventilatory drive

Euthermic golden mantled ground squirrels exhibit a brisk hypoxic ventilatory response, and a dampened hypercapnic response, typical of fossorial species (Tenny and Boggs, 1986). During hibernation, however, McArthur and Milsom (1991a and b) have shown that ventilatory responses to both O_2 and CO_2 decrease disproportionately. When these responses are normalized to account for the reductions in resting ventilation and metabolic rate, there is a large decrease in the relative sensitivity to hypoxia but a significant increase in the relative sensitivity to hypercapnia. In addition continuously breathing euthermic ground squirrels respond to hypoxia and hypercapnia by modifying the durations of inspiration and expiration to influence breathing frequency and the tidal volume of individual breaths. During episodic breathing in hibernation, however, the length and frequency of breathing episodes change in response to these stimuli while the individual breaths remain relatively constant (Milsom et al. 1986; McArthur and Milsom, 1991a, b; Harris and Milsom, 1995). These observations suggest that there is also a significant change in the control of the components of the breathing pattern associated with the transition between euthermia and hibernation.

1.3. Breathing pattern changes during hibernation.

Very little is known about the mechanisms responsible for the transition from continuous to episodic breathing associated with entrance into hibernation. Why doesn't breathing simply slow and individual breaths become progressively further apart ? Interestingly, there are two conditions where breathing does occur with slow, evenly spaced breaths. Golden-mantled ground squirrels hibernating at ambient temperatures ranging down to approximately 5 °C normally breath in distinct episodes. If ambient temperatures are decreased

to 2 °C breathing episodes are replaced by evenly spaced breaths, although f_R and V_E are unchanged (Milsom, 1988). In addition, squirrels hibernating at an ambient temperature of 5 °C, quickly replace breathing episodes with evenly spaced single breaths following exposure to 1% halothane in air, without any change in body temperature (Milsom et al. in press). In general, both anesthesia and cold depress the function of higher brain centers before influencing lower centers. Based on these generalities, these data are consistent with the hypothesis that breathing episodes are generated by the influence of supramedullary structures which is removed through cooling or anesthesia, resulting in the single-breath breathing pattern (Milsom, 1988; Milsom et al. in press).

1.4. Possible higher centers involved

It is not known which higher neural centers might be involved in the clustering of breaths into episodes during hibernation. In euthermic mammals, however, it is well established that a region of the rostral dorsolateral pons, referred to here as the Parabrachial complex (PBC) and comprised of the medial and lateral Parabrachial and Kölliker-Fuse nuclei, is involved in the modulation of breathing pattern.

The PBC receives input from a wide range of pontine, supra-pontine and medullary areas, as well as receiving spinal input via the solitary tract. The PBC accepts input from the pulmonary vagus and is important in determining aspects of ventilatory pattern such as phase switching between inspiration and expiration and the volume for termination of inspiration (St.John, 1977; St.John and Zhou, 1991; Feldman et al. 1990 q.v.). Thus, the PBC appears to be a processing area where inputs from peripheral receptors and higher centers are integrated and transferred to medullary respiratory centers to then influence the patterning of breathing.

The decreases in temperature associated with entrance into hibernation result in reductions in tissue metabolism and the activity of most (if not all) neural tissues. Cranial EEG activity becomes less distinctive as body temperature decreases during hibernation, and is undetectable below approximately 10° C (Walker et al., 1977). Kilduff et al. (1988 and 1990), using deoxyglucose uptake as a measure of relative activity between distinct neural regions, determined that neural activity was not uniformly depressed during hibernation, however. These authors found that while the metabolic activity of the forebrain and midbrain was generally depressed, a number of brainstem nuclei had relatively elevated activities (ie. their activity was proportionately less depressed). These included reticular-formation and hypothalamic nuclei associated with the control of euthermic sleep and wakefulness, which were suggested to be involved in the control of hibernation (Kilduff et al. 1990). Nuclei within the pneumotaxic center also exhibited activity that was elevated relative to other nuclei, suggesting that the influence of the PBC is preserved during hibernation. Consequently, in the present study, it was hypothesized that the PBC would retain its involvement in the modulation of breathing pattern during hibernation, and would be involved in the clustering of breaths into episodes.

1.5. Control of breathing pattern

1.5.1. Central rhythm generation

A primary goal of studies on respiratory control has been identification of the mechanisms responsible for generating breathing pattern. At the forefront of this debate has been the determination of the relative roles of central versus peripheral mechanisms in establishing respiratory rhythm, and shaping this rhythm into ventilatory patterns.

Most early workers assumed the respiratory rhythm was somehow generated in the central nervous system. In 1923, Lumsden demonstrated that normal breathing (eupnea) persisted following the removal of higher neural areas by midcollicular decerebration, but was replaced by a pattern of prolonged inspiration and brief, forceful expiration (termed apneusis) following mid-pontile transection. Lumsden concluded that eupnea resulted from the action of a "pneumotaxic center" located in the rostral pons. Following removal of the pneumotaxic center, ventilation, he proposed, was generated by an "apneustic center" located in the caudal pons. Ponto-medullary transection removed the apneustic center and ventilation then reflected the activity of a medullary "gasping center". Lumsden proposed that each center functioned in an autonomous manner but that the activity of the higher pneumotaxic center superceded that of the lower apneustic which superceded that of the gasping centers. Stella (1938) later identified that apneusis occurred, following removal of the pneumotaxic center, only in the absence of vagal feedback, indicating that either the pneumotaxic center or vagal feedback were necessary for eupnea.

In 1938, Barcroft proposed that (cited in St.John, 1996) the lowest center represented a "kernel" whose activity was modified by the addition of higher influences, such that gasping, apneusis and eupnea each represent the activity of the "kernel" when various higher influences were absent or present. He proposed that the "kernel" itself did not change as overlying levels of complexity were built up or stripped away (Feldman, 1990 q.v.). Lumsden's chain of independent centers and the "kernel" or "reductionist" hypothesis both contrast with a third, "transformational" hypothesis which has arisen more recently (Feldman, 1990). This hypothesis states that the addition or removal of different factors which influence breathing cause transformations in the system of control such that there is little similarity between the

way the system behaves under one set of conditions and another (Feldman, 1990).

In contrast to the general belief that respiratory rhythm was generated within the central nervous system, Sullivan and co-workers (1978) postulated that peripheral receptor feedback alone could account for the production of this rhythm. This hypothesis was further investigated by Phillipson et al. (1981) using sheep fitted with an extra-corporeal artificial lung. In this preparation, animals exhibited proportional reductions in pulmonary ventilation resulting from the removal of CO_2 from the venous blood. When CO_2 was removed at a rate equal to metabolic production, ventilation ceased. Thus, rhythmic ventilation was suppressed when chemoreceptor-mediated ventilatory demand was satisfied. The authors concluded, therefore, that respiratory rhythm generation was critically dependant on chemoreceptor stimuli.

Subsequently, Suzue (1984), using an *in vitro* isolated neonatal rat brain stem-spinal cord preparation, obtained rhythmic discharge from phrenic, hypoglossal and other spinal nerves. The pattern of discharge was altered by changes in pH and depressed by opiates. Suzue postulated that this rhythmic activity corresponded to the respiratory rhythm generated in the intact animal and, as it was expressed in the absence of peripheral inputs and influences from higher neural centers, concluded that a site of respiratory rhythmogenesis was located within the brainstem. These conclusions have been supported by further studies using similar *in vitro* preparations (Feldman et al., 1988; Richter and Spyer, 1990 q.v.; St.John, 1996; Bonham, 1995 q.v.; Bianchi et al.1995 q.v.). Similarly, normal respiratory rhythm persists following decerebration and decerebellation *in vivo* (Feldman et al. 1988 q.v.). Transection of the spino-medullary junction in either preparation abolishes respiratory activity in spinal nerves, while rhythmic activity in cranial nerves is unaffected (Feldman et al. 1988). This more recent data suggests that the brainstem is the site of respiratory rhythmogenesis and that

peripheral inputs act only to modify this innate central rhythmicity.

These contrary findings; observations of a cessation of ventilation in the absence of chemoreceptor drive (Phillipson et al. 1981) and of innate respiratory rhythmicity in the brainstem (Suzue, 1984), can be accommodated if ventilatory rhythm is considered to be the result of a "conditional" central rhythm generator (CRG) (Feldman et al. 1990). With this scheme, the rhythmic activity of the CRG is viewed as being below an active threshold such that the rhythmicity is only expressed at the level of membrane potentials but not as efferent output. The CRG requires additional input to overcome threshold and produce a rhythmic efferent output that will drive pre-motor and motor neurons (see Feldman et al. 1990; Milsom, 1990; Smatresk, 1990 for review). Expression of respiratory rhythm, therefore, is critically dependent on supplementary inputs, although the genesis of respiratory rhythm is central.

The functional properties, exact location and components of the CRG are currently under debate (Bianchi et al. 1995 q.v.). Smith et al. (1991), using systematic micro dissection of the *in vitro* brainstem have localized rhythmogenic neurons to the "Pre-Botzinger complex", within the reticular formation of the ventral medulla. Other studies suggest that the genesis of ventilatory activity incorporates elements from a considerably larger area within the medulla and caudal pons (St.John, 1996 q.v.). Regardless of the exact location, it appears that specific areas within the brainstem are critical for the genesis of respiratory rhythm and, thus, comprise a CRG.

Mechanistically, CRG rhythmogenesis has been attributed to the action of pacemaker cells (Smith et al. 1991; Feldman et al. 1990 q.v.) or a cellular network (Richter et al. 1986; Ogilvie et al. 1992). Oscillations driven by a conditional pacemaker would result from endogenous membrane properties of specific cells. Network driven oscillations, on the other

hand, would result from inhibitory and excitatory connections between groups of cells. These hypothesized mechanisms are not mutually exclusive, however, since a pacemaker could play an integral role in a larger rhythmogenic network (Smith et al. 1991; Feldman et al. 1990 q.v.; Ogilvie et al. 1992).

1.5.2. Central pattern generation

Respiratory rhythm refers to the output of the CRG. This CRG output is shaped and modified to produce ventilation. Such modifications create patterned breathing. Consequently the structures which cause the modification of CRG output are considered part of a central pattern generator (CPG) (Feldman et al. 1988 q.v.; Bonham, 1995 q.v.). The CPG is a functional concept rather than an anatomically distinct region; it comprises a diffuse series of neural structures which together shape CRG output into breathing pattern.

Extensive neuroanatomical and electrophysiological investigations of brainstem regions connected to respiratory motor centers have identified three distinct clusters of neurons with respiratory related discharge patterns which likely represent components of the CPG (see Feldman, 1986; Bianchi, 1995; Richter and Spyer, 1990 for review). Two such clusters occur within the medulla and are referred to as the dorsal respiratory group (DRG) and ventral respiratory group (VRG). The DRG corresponds to the ventrolateral region of the solitary tract, and the VRG comprises the nucleus ambiguus-retroambiguualis extending from the rostral cervical spinal cord through the medulla to the retrofacial nucleus (Feldman et al. 1988 q.v.). The Pre-Botzinger complex, hypothesized to include the CRG, extends from the caudal end of the retrofacial nucleus and the rostral VRG toward the lateral reticular nucleus (Smith et al. 1991).

The third cluster of respiratory-related neurons is located in the rostral dorsolateral pons within the medial and lateral Parabrachial and Kölliker-Fuse nuclei. This region has been referred to as either the pneumotaxic center (Lumsden, 1928, St.John, 1990 q.v.) or the pontine respiratory group (Feldman, 1986 q.v.). These two terms are not interchangeable as each carries with it an assumed function. The pneumotaxic center is an element of Lumsden's chain of independent functional "centers" (St.John, 1990 q.v.), while the pontine respiratory group is a component acting upon Barcrofts's kernel (Feldman, 1986 q.v.). Arguments for and against the use of each term (Feldman, 1986; St.John, 1990 q.v.) suggest that a totally appropriate term has yet to be put forward. This undoubtedly reflects our incomplete understanding of the function of this complex and the drawbacks associated with function-based nomenclature . Given this, the present investigation will refer to the region of the pons which includes the Parabrachial and Kölliker-Fuse nuclei by an anatomical designation, the Parabrachial complex (PBC).

1.5.3. Mechanoreceptor influences on pattern

Respiratory movements are monitored by pulmonary mechanoreceptors activated by changes in lung volume, and by muscle spindles and tendon organs of the chest wall and diaphragm. Afferent fibers from pulmonary receptors project to the brain through the vagus nerve and feedback to respiratory centers in both the medulla and pons (see Coleridge and Coleridge, 1986 for review). There are three primary types of pulmonary receptors in mammalian lungs; rapidly-adapting receptors (RARs), irritant-receptors (C-fibers) and slowly-adapting pulmonary stretch receptors (PSRs). Of these receptor types only the PSRs are typically involved in ventilatory control. RARs and C-fibers are primarily sensitive to lung

irritation and are involved in defense reflexes (Coleridge and Coleridge, 1986 q.v.).

Vagal feedback from PSR's provide both phasic and tonic inputs to respiratory centers. These two input types, and the timing of the phasic inputs, influence respiratory patterning in different ways. Phasic activity reports the timing and volume of each lung inflation and is superimposed on tonic vagal activity which codes information regarding the inflation state of the lung at the end of the breath (i.e. the functional residual capacity or resting lung volume).

The most commonly recognized influence of phasic vagal feedback on breathing is the termination of inspiration, via the Breuer-Hering inspiration-termination reflex (Breuer, 1868; see Coleridge and Coleridge, 1986 for review). The magnitude of vagal stimulation necessary to produce a termination of inspiration falls as a breath progresses, such that inspiratory-termination occurs when the progressively increasing vagal feedback meets the declining threshold of an "intrinsic" off-switch mechanism (Von Euler et al. 1973; Bradley et al.1975). Phasic vagal feedback acts primarily to stabilize resting breathing pattern, yet has differential influences depending on the phase at which it occurs. Phasic activity during the first 30% of inspiration has no effect, whereas during the balance of the inspiratory phase inspiration is enhanced through a low-threshold, volume-dependent facilitatory reflex (Feldman and Gautier, 1976; Cross et al. 1980). Phasic input arriving during the last phase of inspiration is inhibitory to inspiration. Finally, phasic input during early- and mid-expiration stimulates expiration, but input arriving during late expiration has no effect. These influences result exclusively from volume-related activity, while the rate of inflation has no effect (Cross et al. 1980).

Tonic vagal feedback primarily regulates the duration of expiration and the end-expiratory pause, and has little or no influence on either the inspiratory duration or tidal volume (Phillipson, 1974; Coleridge and Coleridge, 1986 q.v.). Decreases in tonic vagal feedback

generally result in a decrease in the duration of expiration and an increase in breathing frequency while increases in tonic feedback are associated with increases in the duration of expiration (Knox, 1973; Trippenbach et al. 1985). These reflexes generally act to match fluctuations in the durations of inspiration and expiration and to modify the volume of expiration and preserve lung volume at functional residual capacity (Trippenbach et al. 1985; Coleridge and Coleridge, 1986 q.v.). Vagotomy generally results in an increase in tidal volume and a decreases in breathing frequency. The increase in tidal volume is to be expected by the loss of phasic vagal feedback. The decrease in breathing frequency, however, results from a prolongation of both inspiration and expiration. Decreases in phasic vagal feedback are associated with prolongations of inspiration, while decreases in tonic vagal feedback produce decreases in the duration of expiration and increases in breathing frequency. Thus, the prolongation of expiration following the loss of all vagal feedback is not what would be expected from decreases in tonic or phasic inputs. It appears that there is an additional aspect of vagal feedback which influences breathing frequency by influencing the duration of expiration (and the end-expiratory pause between active expiration and inspiration) such that vagal stimulation increases f_R and vagotomy generally depresses f_R (Phillipson, 1974; Sullivan et al. 1978; DiMarco et al. 1981; Fedorko et al. 1988; Coleridge and Coleridge, 1986 q.v.).

1.5.4. *Pontine influences on pattern*

There are extensive reciprocal connections between medullary respiratory groups and supra-medullary centers. The most prominent of these is the PBC. Neurons within the PBC exhibit respiratory related activity patterns, and stimulation or lesion of this areas has descending influences on breathing (see Feldman et al. 1988; Richter and Spyer, 1990 for

review).

The pattern of activity of pontine respiratory neurons is primarily tonic, whereas medullary units exhibit phasic activity patterns (Lydic and Orem, 1979; Sieck and Harper, 1980; Dick et al., 1994). Although activity in the PBC is primarily tonic these units receive both phasic and tonic inputs (Dick et al. 1994). Phasic inputs to the pons arise from pulmonary afferents and reciprocal connections between the pons and phasicly active medullary respiratory groups (Lydic and Orem, 1979; Feldman and Gautier, 1976; Dick et al. 1994). Feldman and Gautier (1976) reported that respiratory-related rhythmicity of pontine units was increased by removal of vagal input or prevention of lung inflation. These results suggest that phasic vagal input normally balances the ascending phasic activity of medullary inputs, resulting in tonic output from pontine cells. Tonic input to the pons has been attributed primarily to activation or deactivation by tegmental, reticular and cerebellar mechanisms (Lydic and Baghdoyal, 1993; Dick et al. 1994; Gilbert and Lydic, 1994; Hayes et al. 1994).

Of particular interest to the present investigation is the contribution of the PBC to the inspiratory off-switch mechanism. Cohen (1971) and Bertrand and Hugelin (1971) established that inspiratory and expiratory phase switching mechanisms could be activated by electrical stimulation within the PBC. Von Euler and Trippenbach (1975) determined that the magnitude of stimulation necessary to produce a termination of inspiration fell as a breath progressed and, thus, that the inspiratory off-switch mechanism mediated by the PBC followed the same time course as that mediated by vagal afferent feedback. They concluded that the inspiratory off-switch mechanism could be activated by either vagal feedback or stimulation of the PBC. Many subsequent studies have illustrated that the PBC can directly influence the termination of inspiration (see Bianchi et al. 1995; Feldman, 1986; Bonham, 1995 for review).

Autoradiographic and immunocytochemical surveys (Monaghan and Cotman, 1985; Petralia et al.1994) have shown that the PBC contains a high proportion of the N-methyl-D-aspartate (NMDA)-type receptor for the excitatory amino acid glutamate, and that this receptor type is otherwise relatively scarce in midbrain and brainstem regions. Functions of the PBC associated with ventilation are mediated through NMDA-type receptors, whereas the rhythmogenic properties of the CRG and reflex ventilatory responses such as the Breuer-Hering reflex do not involve NMDA-mediated processes (Greer et al. 1991; Funk et al. 1993; Bonham, 1995 q.v.). Disruption of the function of NMDA-type receptors with specific antagonists has, thus, been used to investigate NMDA-mediated influences of the PBC in many species (Connelly et al. 1992; Ling et al. 1993; Cassus-Soulanis et al. 1995; Bonham, 1995 q.v.).

1.5.5. *Pontine and mechanoreceptor interactions*

The influences of both vagal afferent information and the PBC on breathing pattern appear similar and synergistic (see Feldman, 1986; Bianchi et al. 1995 for review). Vagal input depresses both inspiratory and expiratory time. Removal of vagal inputs result in increases in inspiratory and expiratory duration with consequent increases in tidal volume and decreases in breathing frequency. Pontine input, too, depresses inspiratory and expiratory duration and removal of pontine input has a similar influence to vagotomy. Thus, the pons and vagus are believed to be roughly redundant in their influence on breathing. One or the other is necessary to stabilize breathing (Feldman, 1988 q.v.). Profound disruption of breathing pattern generally results if PBC influences are interrupted in conjunction with an absence of afferent vagal inputs. The result is the breathing pattern, consisting of abnormally prolonged inspirations

interrupted by short expirations, referred to as apneusis. This disruption has been characterized most closely in cats but has been observed in many other mammalian species (Cassis-Soulanis et al. 1995; see also Feldman, 1988; Bianchi et al.1995 for review). Wang et al. (1993) have suggested that the rodent respiratory control network is identical to that of the cat. Rats, however, have not been demonstrated to exhibit apneusis as readily as have cats and conflicting reports suggest that blockade of NMDA receptor-mediated processes in PBC neurons in conjunction with vagotomy may not produce apneusis in all strains of rat (Montreau et al. 1989; Montreau et al. 1990; Connelly et al. 1992; Jodkowski et al. 1994, Wang et al. 1994; Cassus-Soulanis et al. 1995). These conflicting reports rase a host of questions concerning the relative importance of NMDA and non-NMDA receptor-mediated pontine influences in the control of breathing pattern, as well as the confounding influences of specific anesthetics, or species and strain on the role of the PBC in the production of breathing pattern (Connely et al. 1992, Cassus-Soulanis et al. 1995).

1.5.6. Chemoreceptor influences on pattern

Respiratory movements result in lung ventilation, the end result of which is gas exchange between air and blood within the lung. Gas exchange is monitored by peripheral chemoreceptors, located in the carotid and aortic bodies, which are sensitive to levels of O_2, CO_2 and pH in arterial blood (Heymans, 1930; Comroe, 1939). Chemoreceptor afferents project to sensory nuclei in the medulla via cranial nerves. The afferent fibers of the carotid bodies project via the glossopharyngeal nerve, and those of the aortic bodies project via the vagus nerve (see Fitzgerald and Lahiri, 1986; Fidone and Gonzalez, 1986; Smatresk, 1990 for review). Of the two, the carotid bodies have the greater influence on ventilation and respond

primarily to changes in the partial pressure of both O_2 and CO_2. Aortic bodies, in contrast, are less important to the maintenance of ventilation or generation of hypoxic ventilatory responses and are more sensitive to correlates of O_2 delivery, contributing to cardiovascular reflexes (Lahiri, 1980). While carotid body chemoreceptors respond to CO_2, they are fundamentally responsible for O_2 sensation, as carotid and aortic body denervation results in the elimination of hypoxic ventilatory responses while hypercapnic ventilatory responses remain essentially intact (St.John, 1977). Hypercapnia is primarily sensed centrally, via populations of chemosensitive cells proximal to the ventral surface of the medulla which react to CO_2 and appear to monitor CO_2/pH levels in the cerebraospinal fluid (CSF) (Issa and Remmers, 1992; Coates et al. 1993; see Fitzgerald and Lahiri, 1986; Fidone and Gonzalez, 1986; Smatresk, 1990 for review). Input from these central chemoreceptors is also integrated within the medulla (St.John et al. 1989; Bianchi et al. 1995 q.v.).

Decreases in O_2 or increases in CO_2 and/or pH will signal an increase in ventilatory demand and prompt an increase in ventilation. The influence of these stimuli on breathing pattern, however, is different. There is a great deal of variation between the ventilatory responses exhibited by different species. This variation can be attributed to adaptation to different environmental, behavioral and physiological demands. In awake, continuously breathing mammals such as ground squirrels, however, hypoxia will typically increase ventilation through increases in both tidal volume and respiratory frequency, whereas ventilatory increases associated with hypercapnia result primarily from increases in tidal volume (Tenny and Boggs, 1986; Milsom, 1990b).

The PBC is involved in the integration of central and peripheral chemoreceptor afferent stimuli and has been shown to influence both the hypoxic and hypercapnic ventilatory

responses (St.John, 1977). Disruption of the PBC interferes with the frequency component of these responses, while the tidal volume component is retained (St. John, 1977; Fung and St.John, 1994). NMDA receptor-mediated processes are involved in ventilatory chemoresponses. Treatment with NMDA antagonists attenuates or blocks the increases in ventilation normally associated with hypoxia (Kubo et al. 1993; Soto-Arape et al. 1995; Lin et al. 1996; Ang et al. 1992; Chae et al. 1993; Miyawaki et al. 1994). This results from the interruption of integration of afferent input from the carotid body chemoreceptors at the level of the nucleus tractus solitarii (Kubo et al. 1993; Vardhan et al. 1993; Ogawa et al. 1995; Mizusawa et al. 1994; Haxhiu et al. 1995), the phrenic nucleus (Chitravanshi and Sapru, 1997), and the rostroventrolateral reticular nucleus (Sun and Reis, 1995). NMDA receptor also contribute to the ventilatory response to hypercapnia. Interruption of a population of these receptors located within the retrotrapezoid nucleus, decreases the sensitivity to CO_2 as recorded by the phrenic neurogram (Nattie et al. 1993, Nattie and Li, 1995).

Feedback from pulmonary stretch receptors, have been shown to influence ventilatory chemoresponses (Fitzgerald and Lahiri, 1986 q.v.). Vagotomy depresses the changes in breathing frequency associated with hypercapnia (Richardson and Widdicombe, 1969; Phillipson et al. 1973; Martin-Body and Sinclair, 1987) but does not influence the alterations in breathing frequency occurring during ventilatory responses to hypoxia (Chapman et al.1982).

1.6. Synopsis

Work to date suggests that the NMDA receptor-mediated processes within the PBC and vagal afferent feedback have roughly redundant influences on breathing pattern. Both provide inputs to medullary respiratory centers and influence; (i) inspiratory duration and, therefore, tidal volume, and (ii) expiratory duration and, therefore, respiratory frequency. One or the other of these two influences are required for the expression of eupnea, while apneusis results from the absence of both. Some specific rodent strains may not exhibit apneusis following the removal of both of these two influences.

Nothing is known about the involvement of pontine influences and vagal afferent feedback in the generation of episodic breathing patterns during hibernation. There is some data to suggest that episodic breathing result from the influence of higher neural centers. The PBC acts as an integration site for inputs from higher centers and may itself influence breathing pattern. The metabolic activity of the PBC is preserved during hibernation, suggesting that its function, too, may be preserved.

The present series of experiments characterizes the individual and combined influences of afferent vagal feedback and NMDA receptor-mediated processes both within and outside the PBC on the genesis and stability of breathing pattern in the golden mantled ground squirrel. These characterizations are made, where possible, in unanesthetized animals during euthermia and hibernation, and in anesthetized animals and document the influences of these factors on both continuous and episodic breathing patterns.

General Hypothesis:

The general hypotheses examined by this study are that breathing pattern in squirrels is mediated by both vagal and pontine influences, that these influences retain their importance during hibernation and that they underlie the genesis of breathing in episodes.

Specific Hypotheses:

1) Vagal afferent feedback influences the resting breathing pattern of spontaneously breathing golden mantled ground squirrels during sleep and wakefulness, anesthesia and natural hibernation.

2) Vagal feedback influences the ventilatory responses to chemoreceptor stimuli which occur in unanesthetized and anesthetized, euthermic squirrels, and will influence ventilatory responses during the episodic breathing pattern in hibernating squirrels.

3) NMDA receptor-mediated processes influence the resting breathing pattern of spontaneously breathing golden-mantled ground squirrels during sleep and wakefulness, anesthesia and natural hibernation.

4) NMDA receptor-mediated processes influence the ventilatory responses to chemoreceptor stimuli which occur in unanesthetized and anesthetized, euthermic squirrels, and will influence ventilatory responses during the episodic breathing pattern in hibernating squirrels.

5) Vagal feedback and NMDA receptor mediated processes in the pons interact to prevent apneusis in euthermic squirrels during sleep and wakefulness, and anesthesia.

6) Vagal feedback and NMDA receptor mediated processes in the pons interact to produce breathing episodes in hibernating squirrels.

Chapter 2

General Methods

2. General Methods

2.1. Animals

Experiments were performed or attempted on a total of 68 adult golden mantled ground squirrels *(Spermophilus lateralis*, 235 ± 5 g) of mixed sex. These animals were originally collected from a wild population by a supplier in Redding California. They were imported into Canada under permit from the Ministry of Environment, Province of British Columbia and were maintained in a research colony for at least one year before experimentation. Before these studies, animals were housed in pairs in wire-topped polycarbonate cages (24 x 45 x 20 cm) in a sound insulated, climate-controlled chamber at constant ambient temperature (20 ± 1 °C) and photoperiod (12 hours of light and 12 hours of dark; 12L:12D). Squirrels had access to lab chow and water *ad lib.* supplemented intermittently with sunflower seeds and fresh fruit.

2.2. Animal Preparation and Procedures: chronic studies (euthermic and hibernating)

2.2.1. Electrode placement

Animals were anesthetized with intraperitoneal injections of sodium pentobarbital (Somnotol, 65 $mg.ml^{-1}$; 45-65 $mg.kg^{-1}$). It was determined that animals had reached a surgical plane of anesthesia when they became flaccid and no longer exhibited either an eye-blink or limb-withdrawal reflex. Surgery was done under sterile conditions and all tools and equipment were sterilized in an autoclave or by emersion in disinfectant (Germex, MTC Pharmaceuticals).

A series of electrodes were fashioned from lengths of insulated, multi-stranded, stainless steel wire (AM Systems). Gold pins (Amphenol) were soldered to one end of these electrodes and the other end was attached to either a stainless steal washer (size 00, Pacific

Fasteners) (ECG and EMG electrodes) or a self-tapping stainless steel screw (00 x 3/16, Fine Science Tools) (EEG electrodes).

A dorsal-longitudinal incision was made over the crown of the cranium, extending from the orbits to the base of the neck. Four cranial electroencephalographic (EEG), two electrocardiographic (ECG), two electromyographic (EMG) and two respiratory impedance electrodes were implanted in the skull, rib-cage, shoulder musculature and abdominal wall, respectively as described in Hunter and Milsom (1997). Electrode wires were run sub-cutaneously to a 10-pin connector which was cemented to the skull with dental epoxy.

2.2.2. Vagal cuff

An incision was made in the ventral neck and the left and right cervical vagi were isolated. The right vagus was tagged for later identification by encircling it with two 2 cm loops of suture. The left vagus was placed within an "infusion cuff", consisting of a 4 mm length of silicone tubing (0.08" internal diameter) slit down one side, with a silicone cannula (0.02" internal diameter) fastened in the middle of its inside edge. Two loops of suture closed the cuff around the nerve and both ends were plugged with sterile petroleum jelly. Fluid fed down the cannula filled the cuff and bathed the isolated section of nerve. Subsequent infusions renewed the fluid within the cuff while the overflow seeped out of the cuff's slit edge. Previous investigations indicated that a 0.2 ml infusion of the local anesthetic, xylocaine (2% lidocaine hydrochloride, Astra Pharmaceutical) was sufficient to produce a complete nerve block within 30 seconds (Harris and Milsom, 1995). The cuff was secured in position and the cannula fed subcutaneously over the shoulder to a connector mounted to the skull.

Following surgery, the incisions were closed and the animal allowed to recover. Four

to six days following initial surgery, animals were again anesthetized with either an intra peritoneal injection of sodium pentobarbital (Somnotol, 65 $mg.ml^{-1}$; 25-45 $mg.kg^{-1}$), or vaporous halothane (3.5% in air). The incision on the ventral neck was opened and the right vagus was exposed and identified using the previously implanted loops of suture. The right vagus was bathed in xylocaine, severed, and a 5mm section was removed. The incision was closed, and animals were observed as the influences of anesthesia subsided.

2.2.3. Third cerebral ventricle cannulation

Animals were placed in a stereotaxic head frame (Kopf), adjusted such that the skull surface landmarks lambda and bregma were on the same horizontal plane. The third cerebral ventricle was cannulated using the technique of Boswell et al. (1993). A 22-gauge guide cannula (Plastics One, Roanoke VA.) was held in a jig fabricated from 28-gauge steel tubing attached to a micro-manipulator. The tip of the guide cannula was lowered 8.5 mm anterior to the interaural line, and on the midline, through a hole drilled in the skull. Cannula insertion was facilitated by first perforating the meninges and retracting the mid-sagittal sinus laterally. The cannula was dropped 7.0 mm below the cortical surface and anchored to the skull in a dental acrylic "cap". The jig was slowly removed from the guide cannula and, in all cases, cerebrospinal fluid was observed to flow to the top of the open guide cannula confirming placement within the cerebral ventricle. A 28-gauge "dummy" cannula (Plastics One , Roanoke VA.) was inserted into the guide cannula and secured to the threaded flange of the guide by its threaded cap.

2.2.4. Timing of instrumentation

Squirrels selected for the unanesthetized euthermic portions of the study underwent surgery during the summer. They required no further preparation but were held for at least 3 weeks following the second operation before experimentation.

In late November, squirrels selected for the unanesthetized hibernating portion of the study were induced to hibernate by a gradual reduction of chamber temperature (to 5 ± 1 °C) and photoperiod (2L:22D). Most animals entered hibernation within two weeks of exposure to these "winter" environmental conditions. Beginning in mid-February, individual hibernating animals were removed from the environment chamber and held for three days at ambient temperature (20 ± 1 °C) and photoperiod (12L:12D) before undergoing the surgeries outlined above. Following recovery, animals were returned to "winter" environmental conditions (5 ± 1 °C and reduced photoperiod, 2L:22D) and most animals reentered hibernation within two weeks.

2.3. Animal Preparation and Procedures: acute studies (euthermic anesthetized)

2.3.1. Electrode placement and cannulation

Squirrels used in this series of experiments were anesthetized with a 5ml/kg intra peritoneal injection of a 20% solution of Urethane (Sigma, dose =1g/kg) in saline. Supplemental anesthesia, to facilitate surgery, was induced using vaporous Halothane (Wyeth-Ayerst, 3.5% in air) administered via a tight fitting mask. Four cranial electroencephalographic (EEG), two electrocardiographic (ECG) and two electromyographic (EMG) electrodes were implanted in the skull, rib-cage musculature, and shoulder musculature, respectively as described above. A second incision was made ventrally in the neck to implant a tracheal

cannula and expose and isolate the left and right cervical vagi. These nerves were each snared with looped suture passed through a sleeve of narrow polyethylene tubing, to facilitate bilateral vagotomy during the experiment. A third incision was made in the pubic region and catheters were implanted in a femoral artery and vein to allow blood pressure assessment and intravenous infusion of saline and pharmacological agents. Arterial and venous catheters were regularly flushed with sterile saline (0.9% NaCl).

Following instrumentation, halothane administration was discontinued and the animals were placed in an electrically shielded chamber. Body (rectal) temperature was maintained at 36 ± 1 °C using a servo-controlled homeothermic table (Harvard) throughout the experiment. The tracheal catheter was connected (in series) to a ventilatory pneumotachograph attached to the side arm of an air line. Body wall movements associated with ventilation were monitored by respiratory impedance via two silver electrodes (Grass Instruments) secured to shaved patches of bare skin, in opposition across the lateral abdomen. Electrical conductivity was provided by electrophoretic jelly (Grass Instruments). The respiratory impedance measurements illustrated the relative positions of the animals body wall and, therefore, the state of inflation of the chest. As animals were spontaneously ventilating through a tracheal catheter, the impedance measurements were an indirect indication of the state of respiratory muscle activation during any pause in air flow. Animals were visually monitored for approximately two hours as the influences of halothane subsided.

2.3.2. Timing of instrumentation

Squirrels selected for this portion of the study were instrumented on the day of the experiment, and experiments were done in the summer and fall.

2.4. Measurements

Ventilation was measured in unanesthetized euthermic animals using the modified whole body plethysmograph technique of Jacky (1978; 1980; Milsom and McArthur, 1991a). The plethysmograph consisted of a 10 x 10 x 10 cm plexiglass test chamber connected, in parallel, with an identical reference chamber. Both chambers had two ports to allow the flow of humidified gas, and were initially supplied with humidified air at a flow rate of approximately 1.5 liters per minute. Outlet resistance and flow rate were matched between the two chambers. The lids of each chamber had ports to allow connection of a differential pressure transducer (Validyne) between them. The test chamber had an additional port to allow passage of the electrode leads and cannulae. The test and reference chambers were held within a laboratory controlled-environment chamber adjusted to maintain the temperature at approximately 22 to 25 °C. Test chamber temperature was monitored constantly with a digital thermometer. Pressure fluctuations within the test chamber resulted from the warming and expansion of inspired air by the animal. The differential pressure between the test and reference chambers was measured by the pressure transducer. The output from the pressure transducer was amplified directly through a DC amplifier. The resulting tracings were used to calculate the tidal volume of each breath.

The plethysmograph was calibrated during each test. With the animal in the apparatus, a known volume (V_{CAL}; 1 to 1.5 ml) of humidified air was pumped into the test chamber via a small-animal ventilation pump (Harvard). The injection resulted in an increase in test-chamber pressure. This pressure was proportional to the flow of air into the chamber and was, thus, dependant on both the volume and frequency of the injection. The pressure signal was digitally integrated using data analysis software (AT-CODAS, DataQ Instruments) to determine the area

under the pressure signal (A_{CAL}) which was proportional to the injection volume and independent of injection frequency.

Breathing records from each test were integrated to determine the area under the plethysmograph pressure signal (A_{TEST}). Comparing this value to those obtained during calibration allowed the volume required to generate the observed pressure (V_{TEST}) to be determined from the relationship;

$$V_{TEST} = (V_{CAL})(A_{TEST}/A_{CAL}).$$

As animals were supplied with humidified gas, this pressure producing A_{TEST} resulted solely from the expansion of inspired air due to warming from chamber temperature (T_{CH}) to body temperature (T_B). Tidal volume could be determined as that volume which would result in an expansion, V_{TEST}, when raised from chamber temperature (T_{CH}) to body temperature (T_B), using Charles' Gas Law; $V_1/V_2 = T_1/T_2$, where V_1 is the tidal volume, V_2 is the tidal volume plus the expansion volume ($V_2 = V_1 + V_{TEST}$), and T_1 and T_2 are absolute chamber and body temperatures (in °Kelvin) respectively. Algebraic manipulation of the known variables results in an expression solving for tidal volume (V_1);

$$V_1 = (T_1 / T_2)(A_{TEST} / A_{CAL})(V_{CAL}) / (1 - T_1 / T_2)$$

This calibration technique produced results similar to the dynamic calibration technique described by others (Jacky, 1978, 1980; Epstein and Epstein, 1978, 1980). This technique, however, allowed assessment of tidal volume regardless of breathing frequency and, as plethysmograph pressure always returned to baseline values between breaths, it was not necessary to engage corrections outlined by Epstein et al. (1978, 1980) to account for incomplete cooling of end-tidal gas before subsequent inspiration.

Ventilation was monitored in anesthetized and in hibernating animals using a

differential pressure transducer (Valedyne) attached to the resistance pneumotachograph. In hibernating animals the pneumotachograph was connected to a tight fitting mask, while in anesthetized animals it was connected directly to the tracheal cannula. Transducer output was split and amplified directly through a DC amplifier (Grass Instruments) as well as integrated and amplified by an integrating amplifier (Gould), to provide a measure of both ventilatory flow and tidal volume respectively. The pneumotachograph was calibrated at the end of each test. Known volumes (1 to 2 ml) of air were pumped back and forth through the pneumotachograph via a small-animal ventilation pump (Harvard). The resulting output from the pressure transducer was proportional to flow through the pneumotachograph and increased with pump frequency. The integrated output from the pressure transducer was frequency-independent and proportional to the volume of the calibration injection.

Body wall movements associated with ventilation were monitored in all preparations using a respiratory impedance converter (Biocom inc., model 991) and DC amplifier. The EEG, EMG and ECG were monitored using AC amplifiers (Grass Instruments). All signals were recorded continuously on both a polygraphic recorder, and computerized data acquisition system (AT-CODAS, DataQ Instruments) sampling at either 100, 80 and 40 Hz per channel during studies of unanesthetized, anesthetized and hibernating animals respectively. Sampling frequency was dictated by the duration of the experiments and limited by the storage capacity of the computer running the data acquisition system.

2.5. Experimental treatments

Experimental treatments varied from study to study and are presented separately in each chapter of this thesis.

2.6. Data analysis

2.6.1. Arousal state

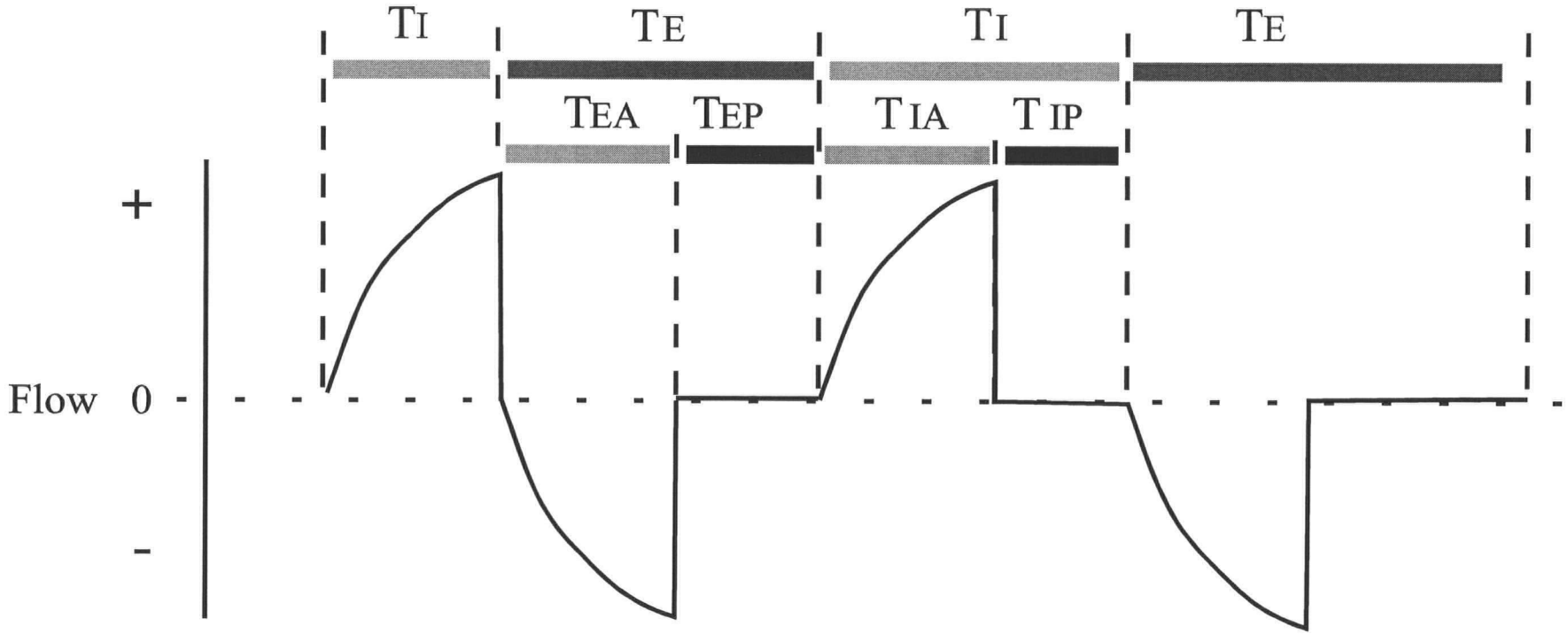
Arousal or activation states in euthermic, anesthetized and unanesthetized animals were determined through subjective examination of EEG and EMG records using established electrophysiological criteria for determination of sleep and wakefulness (Reschtaffen et al. 1968). Secondary analysis of EEG waveforms were achieved by frequency analysis using post-hoc analysis software associated with the computer data acquisition system (ADVPOST and WindaQ, DataQ Instruments).

2.6.2. Euthermic unanesthetized animals

Ventilation in unanesthetized animals was measured via a whole body plethysmograph and by respiratory impedance. Respiratory frequency (f_R) and tidal volume (V_T) were measured during 60-second periods of stable breathing under each treatment condition using post-hoc analysis software. Ventilation (V_E) was calculated as the sum of tidal volumes occurring over each 60 second period. The timing components of each breath during this 60 second period were also measured. Since pauses could occur following either inspiration or expiration, both phases were subdivided into periods during which air flow occurred (T_{IA} and T_{EA}, for inspiration and expiration respectively) and the pauses during which no air flow occurred (T_{IP} and T_{EP} respectively) (Fig. 2.1). Measurement of f_R, V_T, and T_{IA} and T_{IP} could be obtained easily from the plethysmograph records. Precise identification of the termination of airflow in expiration was problematic, however, and thus values reported for T_{EA} and T_{EP} are less precise. The consequences of sleep and wake states and of experimental treatments were assessed by comparing the values obtained using one-way repeated measures analysis of

Figure 2.1.

An illustration of the timing components of each breath. Breaths are divided into phases of inspiration (TI) and expiration (TE). Since pauses could occur following either inspiration or expiration, both phases were subdivided into periods during which air flow occurred (TIA and TEA, for inspiration and expiration respectively) and the pauses during which no air flow occurred (TIP and TEP respectively).


TI
TE
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TE
TEA
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TIA
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variance, Friedman repeated measures analysis of variance on ranks, or two-way repeated measures analysis of variance, where appropriate (SigmaStat, Jandel Scientific). Additional pairwise multiple comparison procedures were done using the Student-Newman-Keuls method. All tests employed an alpha value of 0.05 (or 0.10 where specified), thus, normality, equality of variance and significance of differences were attributed to $P < 0.05$ unless otherwise specified.

2.6.3. Anesthetized euthermic animals

Measurement of f_R, V_T, T_{IA}, T_{IP}, T_{EA} and T_{EP}, and calculations of V_E, were made from representative 60-second traces of stable breathing during states with sleep-like and wake-like EEG patterns, before and after experimental treatments. Data were obtained from the integrated output of the tracheal pneumotachograph, using the post-hoc data analysis software. The effects of changes in state and treatment were assessed by comparing the values obtained using one-way or two-way repeated measures analysis of variance and Student-Newman-Keuls pairwise multiple comparison (SigmaStat, Jandel Scientific). Again, normality, equality of variance and significance of differences were attributed to $P < 0.05$ unless otherwise specified.

2.6.4. Hibernating animals

During hibernation, animals exhibited an episodic breathing pattern. Representative segments of data, consisting of at least 45 minutes of continuous recording, were selected for each treatment condition. These segments were bounded at each end by the termination of a final breath in an episode. Individual breaths were analyzed to determine tidal V_T, and the durations of inspiration (T_{IA} and T_{IP}), expiration (T_{EA} and T_{EA}) and the non-ventilatory pauses

between episodes of breathing (TAPN). Breathing episodes were analyzed to determine the number of breaths per episode, the frequency of episodes, and the instantaneous breathing frequency of the breaths within each episode ($f_{R_{inst}}$ = 60/breath-to-breath interval of each breath in the episode). The overall respiratory frequency (f_R = total number of breaths/duration of the segment) and ventilation volume ($\dot{V}_E$= sum of tidal volumes of each breath/duration of the segment) were also determined from each segment. The effects of each treatment were determined by comparing the values obtained before and after treatment using one-way or two-way repeated measures analysis of variance and Student-Newman-Keuls pair-wise multiple comparisons with alpha values set at 0.05 (SigmaStat, Jandel Scientific).

Chapter 3

The Influence of Vagal Feedback on the Breathing Pattern of Ground squirrels

Abstract

The influence of vagal afferent feedback in terminating inspiration and modulating breathing pattern was assessed in the golden-mantled ground squirrel, *Spermophilus lateralis,* during sleep and wakefulness, anesthesia, and natural hibernation.

While unanestheatized animals cycled between sleep and wakefulness, anesthetized animals cycled between states with cortical activity patterns similar to sleep and wakefulness. Tidal volume increased and breathing frequency decreased as animals cycled from awake and "wake-like" to sleep and "sleep-like" states, respectively. Vagotomy resulted in an increase in tidal volume, a decrease in breathing frequency and ventilation, and abolished the changes in ventilation associated with changes in cortical activity patterns in anesthetized animals. In unanesthetized animals vagotomy produced a "gasp-like" breathing pattern that progressed to a non-obstructive central apnea. Hibernating animals breathed in distinct multi-breath episodes separated by prolonged apneas. Removal of vagal feedback did not alter breathing on a breath-by-breath basis, but did decrease the number of breaths in each breathing episode and depressed overall ventilation.

Thus, while vagal feedback only modulates breathing pattern during natural hibernation, it shapes ventilation on a breath-by-breath basis during anesthesia and is essential for ventilation in unanesthetized animals. The cause for the transformation of the influences of vagal input on breathing under these different conditions remains unclear.

3.1. Introduction

It is generally accepted that the basic rhythm of breathing in mammals is generated within the brainstem through the activity of a "central rhythm generator" (CRG) that can function independent of modulatory inputs (see Bianchi et al. 1995 for review). This basic rhythm, however, is shaped by various inputs to create complex breathing patterns (Feldman et al. 1990 q.v.; Milsom, 1990 q.v.). The CRG and its modulatory inputs act as a functional "central pattern generator" (CPG) and determine the duty cycle, timing of inspiration and expiration, amplitude of the breath, and effectively match ventilation to ventilatory demands.

Afferent information from pulmonary stretch receptors, which constantly monitor the inflation state of the lung, are an important input shaping breathing pattern between and during each breath (see Bianchi et al. 1995; Milsom, 1990 for review). Feedback from pulmonary stretch receptors is carried by afferent fibers running within the vagus nerve which connect monosynaptically and polysynaptically with brainstem nuclei comprising elements of the CPG as well as the CRG itself (See Feldman et al. 1990; Coleridge and Coleridge, 1986 fro review).

The most commonly recognized influence of vagal feedback on breathing is to prompt the termination of inspiration; the Breuer-Hering inspiration-termination reflex. Vagal feedback, however, also contributes to inspiratory and expiratory facilitation and deflation reflexes (Breuer, 1868; Cross et al. 1980; Coleridge and Coleridge, 1986 q.v.). Furthermore, the magnitude of vagal reflexes differs between species (Widdicombe, 1964; Guz, et al.1970), between neonates and adults (Gaultier and Mortola, 1981; Fedorko et al. 1988), and between sleep, wakefulness and anesthesia (Martin-Body and Sinclair, 1987; Hamilton et al. 1988).

The Breuer-Hering inspiration-termination reflex is blocked in mammals by cooling the cervical vagus to between 8 and 14°C (Karczewski and Widdicomb, 1992). During hibernation

ground squirrels regularly maintain body temperatures below this level and it has been generally believed that vagally mediated reflexes are absent during hibernation (Lyman, 1982). Recently, however, it was demonstrated that vagal efferent output contributes to the coordination of heart rate and breathing during hibernation (Harris and Milsom, 1995) raising questions about the role of vagal afferent feedback in controlling breathing pattern during hibernation at low body temperatures.

Given that nothing is known about vagal reflexes and breathing in hibernating species, and the host of factors that can affect the magnitude of these reflexes, the present investigation was designed to assess the influences of vagal afferent feedback on the termination of inspiration and generation of breathing pattern in spontaneously breathing golden mantled ground squirrels during unanesthetized sleep and wakefulness, anesthesia and natural hibernation.

3.2. Methods

3.2.1. Instrumentation

Experiments were performed on a total of twenty six adult golden-mantled ground squirrels *(Spermophilus lateralis)*. Animals used in the chronic studies were surgically instrumented with four electroencephalographic (EEG), two electrocardiographic (ECG), two electromyographic (EMG) and two respiratory impedance electrodes, one vagus nerve was fitted with an infusion cuff and the other vagus was sectioned as outlined in the general methods section (chapter 2).

3.2.2 Protocol

3.2.2.1. Euthermic animals (chronic study)

Six squirrels were selected for this study. For experimental runs, animals were transferred to a whole body plethysmograph. The cannula supplying the vagal cuff was connected to a 3 cc. syringe via a length of polyethylene tubing (PE 50). The syringe and tubing were filled with xylocaine except for the final 0.2 ml at the tip which was filled with saline, separated from the xylocaine by a small air bubble.

Following a two hour stabilization period, the animals were monitored; i) for one hour before any manipulation, ii) for one hour following a 0.2 ml infusion of saline (sham) into the vagal cuff, and then iii) following infusion of 2% xylocaine (vagal blockade) into the vagal cuff.

A single animal was anesthetized with halothane and a ventral incision was made to expose the trachea and cervical vagi. This animal was fitted with a tracheal catheter to bypass the upper airway. The left and right cervical vagi were severed and the incision was then closed, halothane anesthesia was discontinued, and the animal was placed within the whole-body plethysmograph.

3.2.2.2. Hibernating animals (chronic study)

Twelve animals selected for this portion of the study underwent surgery during the winter. Following recovery, these animals reentered hibernation. During deep hibernation, animals were transferred to a plexiglass box (12 x 20 x 10 cm) within a laboratory controlled-environment chamber. Each box was supplied with air at a flow rate of 500 ml per minute. Ambient temperature was held at 5 °C. Animals were fitted with a mask connected to a

resistance pneumotachograph to measure ventilation. The cannula supplying the vagal cuff was connected to a xylocain-filled tube and syringe as outlined above.

Animals were monitored; i) for at least two hours to provide initial control values, ii) for at least one hour following a 0.2 ml infusion of saline (sham treatment) into the vagal cuff, iii) for at least two hours following a 0.2 to 0.4 ml injection of xylocaine (vagal blockade) into the vagal cuff, and iv) for at least one hour following an additional 0.2 ml injection of xylocaine into the vagal cuff.

3.2.2.3. Anesthetized animals (acute study)

Seven ground squirrels were used in this series of experiments. Animals were anesthetized, tracheotomized, fitted with EEG, ECG, EMG and respiratory impedance electrodes and had their left and right cervical vagi isolated, as described in Chapter 2.

Animals were monitored; i) for at least 2 hours before, and ii) for at least two hours following bilateral cervical vagotomy. Vagotomy was achieved by injecting 0.2 ml of xylocaine into one of the previously implanted polyethelyne sleeves, and then by severing that nerve approximately one minute later by pulling the loop of suture through the sleeve. The contralateral vagus was blocked and severed in the same manner three to four minutes later. Pilot studies indicated that there was no significant effect of unilateral vagotomy, nor was there a difference stemming from the order in which the nerves were severed. Pre-treatment with xylocaine prevented mechanical stimulation of the nerve during vagotomy.

3.3. Results

3.3.1. Euthermic animals

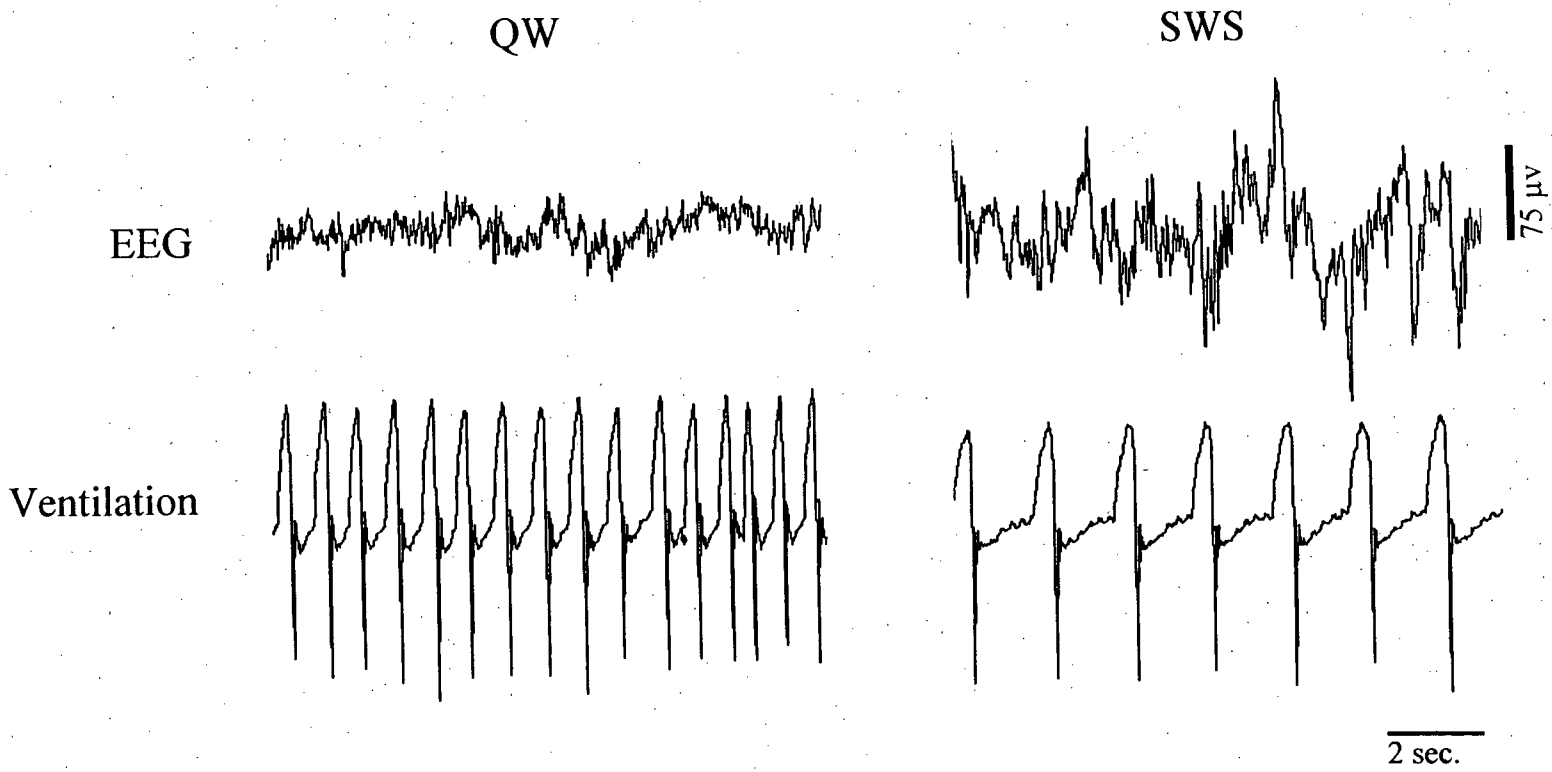
3.3.1.1. Activation state

Unanesthetized animals exhibited distinctive patterns of EEG and EMG characteristic of different phases of vigilance (Fig. 3.1). Wakefulness was characterized by the presence of a high frequency (>6 hz) low amplitude EEG and pronounced postural muscle tone (nuchal muscle EMG). Two phases of wakefulness were noted. One phase, designated as "quiet wakefulness" (QW) where EMG activity was tonic, was easily distinguishable from the other, "active wakefulness" (AW), during which the EMG contained higher amplitude waveforms indicative of more pronounced postural tone, and often contained phasic components associated with activity such as body movement, shivering or locomotion. Slow-wave sleep was characterized by a stereotypical pattern of EEG consisting of high amplitude low frequency (1-4 Hz) activity and diminished muscle tone. Rapid-eye-movement (REM) or paradoxical sleep, characterized by "wake-like" EEG activity and low EMG activity characteristic of postural muscle atonia was also observed. In addition to these, there were a host of poorly established transitional phases between each well established phase.

During these experiments, animals regularly cycled between 10 to 30 minute periods of QW and SWS, interspersed with 5 to 10- minute periods of AW. Although present, REM occurred only in short (<45 second) and irregular bouts. In the present investigation, ventilation was analyzed only during well established periods of QW and SWS. Breathing during AW, REM and the various transition phases was ignored.

Fig.3.1

Representative recordings of EEG and breathing during quiet wake (QW) and slow-wave sleep (SWS). Breathing records are from the output of the plethysmograph, with inspiration as an upward deflection and tidal volume proportional to the area under the deflection.


QW
SWS
EEG
Ventilation
75 µv
2 sec.

3.3.1.2. Ventilation

Animals exhibited an increase in tidal volume (V_T) and decrease in respiratory frequency (f_R) as they progressed from QW to SWS (Fig. 3.2). There was a slight but non-significant reduction in ventilation (V_E) between these states. Changes in breathing pattern resulted from increases in inspiratory (T_I) and expiratory (T_{EA}) durations, and the durations of end-expiratory pauses (T_{EP}) between states (Fig. 3.3).

3.3.1.3. Vagal blockade

Sham injections of saline had no effect on the expression of activation states or on ventilation. Vagal blockade, however, always induced an immediate transition to AW. Within 30 seconds of the initiation of vagal blockade, spontaneous ventilation ceased and animals exhibited a prolonged period of apnea. This apnea was punctuated by isolated events that on viewing the animal appeared to be "gasp-like" breaths. If allowed to progress, the frequency of "gasp-like" breathing decreased and spontaneous ventilation ceased. Animals were immediately removed from the plethysmograph, the vagal cuff was flushed with saline and animals were artificially ventilated in an attempt to restore spontaneous ventilation. In 3 of 6 animals, artificial ventilation was initiated immediately following the induction of vagal blockade, before ventilatory arrest and "gasp-like" breathing began. Spontaneous ventilation was restored in only 2 of 6 animals, and only after at least 30 minutes of artificial ventilation.

Direct observation and recordings of respiratory impedance suggested these apneas were central and not obstructive. There were no indications of body wall movement and attempted breathing. To further assess the possibility that upper airway occlusion led to gasping and ventilatory arrest in these animals, a single animal was tracheotomized to bypass the upper

Fig. 3.2

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and minute ventilation (V_E) in; unanesthetized animals during quiet wake (open bars) and slow-wave sleep (hatched bars) (left panels), and anesthetized animals during wake-like (open bars) and SWS-like (hatched bars) states (right panels) before (Intact) and after vagotomy (VX). The "*" denotes a significant difference from QW or State-I values within the treatment (either Intact or VX), while the "#" denotes a significant difference from values in the same State (I or III) in intact animals ($P<0.05$).

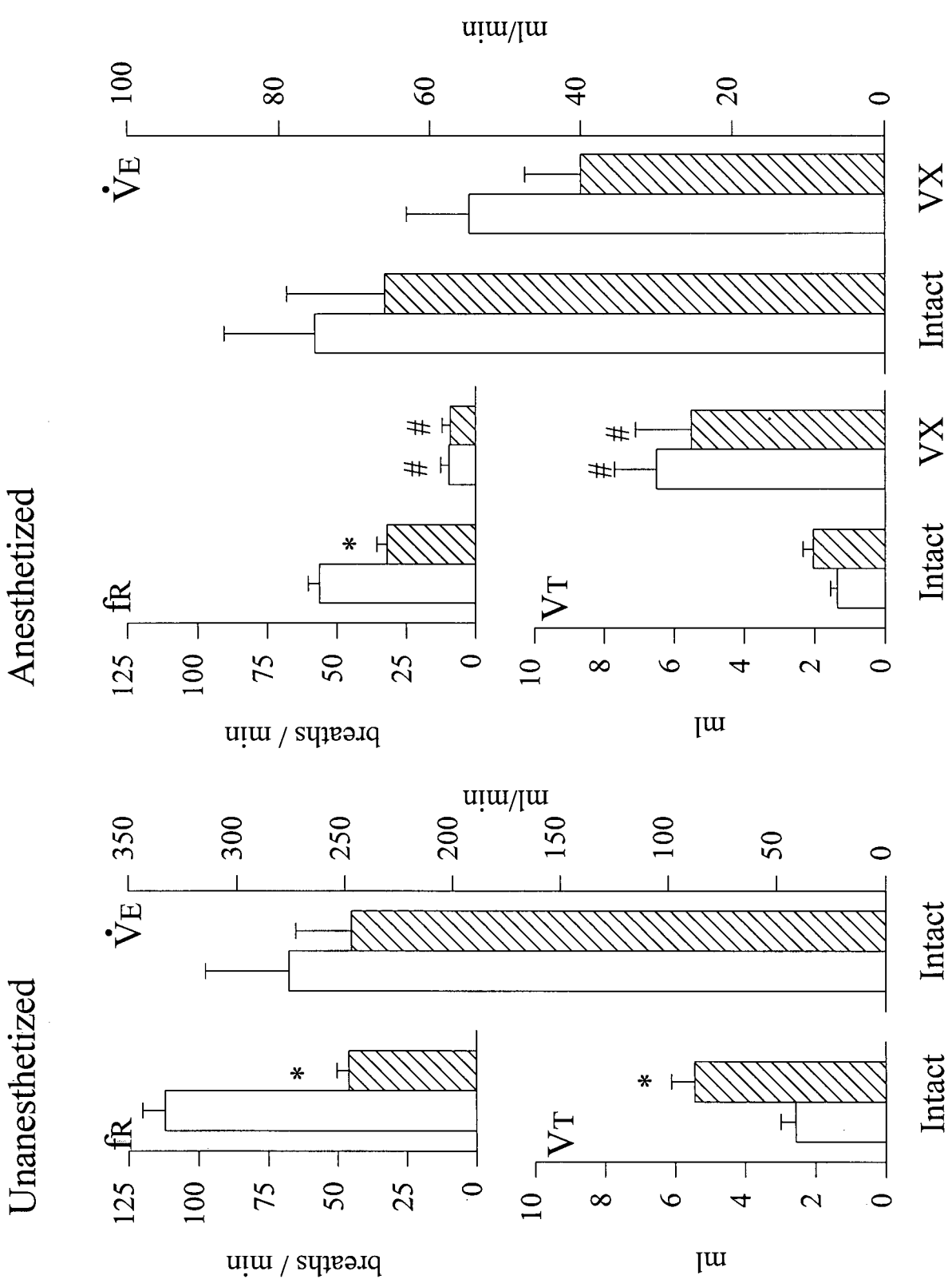
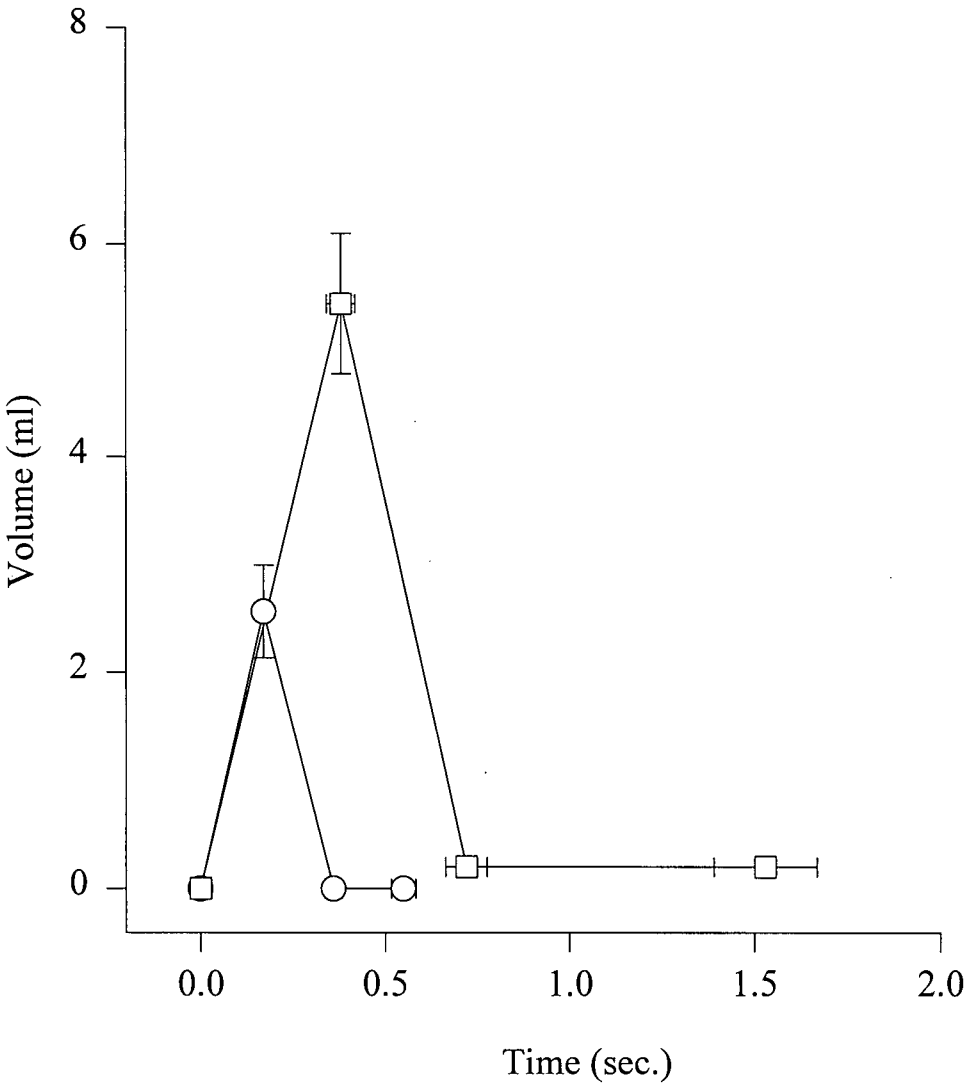

Unanesthetized
Anesthetized
fR
breaths / min
125
100
75
50
25
0
*
$\dot{V}_E$
ml/min
350
300
250
200
150
100
50
0
V_T
ml
10
8
6
4
2
0
Intact
VX
#

Fig. 3.3

Representation of mean (± standard error) inspiratory volumes and durations of inspiration (T_I) and expiration (T_{EA} and T_{EP}) of unanesthetized animals during quiet wake (○) and slow-wave sleep (□). Statistical differences are noted in the text. Note that in different plots the end-expiratory volumes have been offset from zero for clarity.


8
6
4
2
0
Volume (ml)
0.0
0.5
1.0
1.5
2.0
Time (sec.)

airway. While still under anesthesia this animal was vagotomized and continued to breathe spontaneously with a slow and deep pattern of breathing. Anesthesia was discontinued and the breathing pattern rapidly changed and spontaneous ventilation now occurred only in "gasp-like" breaths with decreasing frequency. Eventually spontaneous ventilation ceased. Artificial ventilation with air was begun immediately but did not restore a normal breathing pattern.

3.3.2. Anesthetized animals

3.3.2.1. Activation state

Under Urethane anesthesia, two distinct patterns of EEG were observed (Fig. 3.4). The EEG waveform showed a predominance of either high frequency (>6Hz) low amplitude activity (State-I) or, high amplitude low frequency (1-4 Hz) activity (State-III). In addition, a less common pattern (State-II) also occurred, which contained both wave-forms and resembled a period of transition between the two primary states.

3.3.2.2. Ventilation

Breathing pattern was characterized during each of the activation states (Fig. 3.2). Animals exhibited a decrease in f_R as they progressed from State-I to State-III. There were, however, no significant differences in V_T, or V_E between these states. Decreases in f_R resulted from increases in T_I and T_{EP} between states, T_{EA} was unchanged (Fig. 3.5).

3.3.2.3. Vagotomy

Coincident with vagotomy, animals in States II or III moved into State I. Following vagotomy, however, animals subsequently continued to cycle between States I, II and III.

Fig. 3.4

Representative recordings of EEG and breathing during State-I and State-III in anesthetized animals before (top) and after (bottom) vagotomy. Breathing records are the integrated output of the pneumotachograph with inspiration as an upward deflection and peak height proportional to tidal volume.

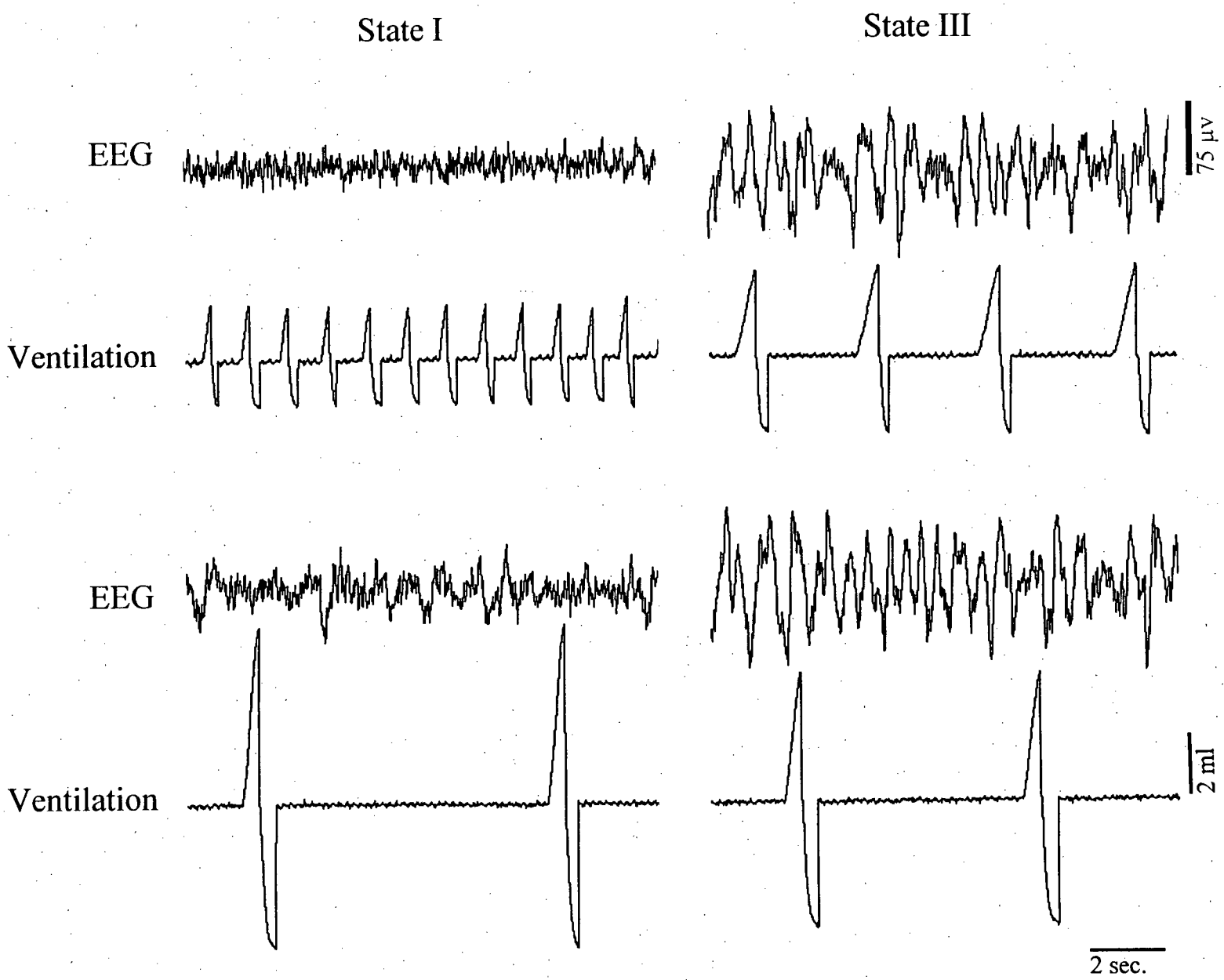
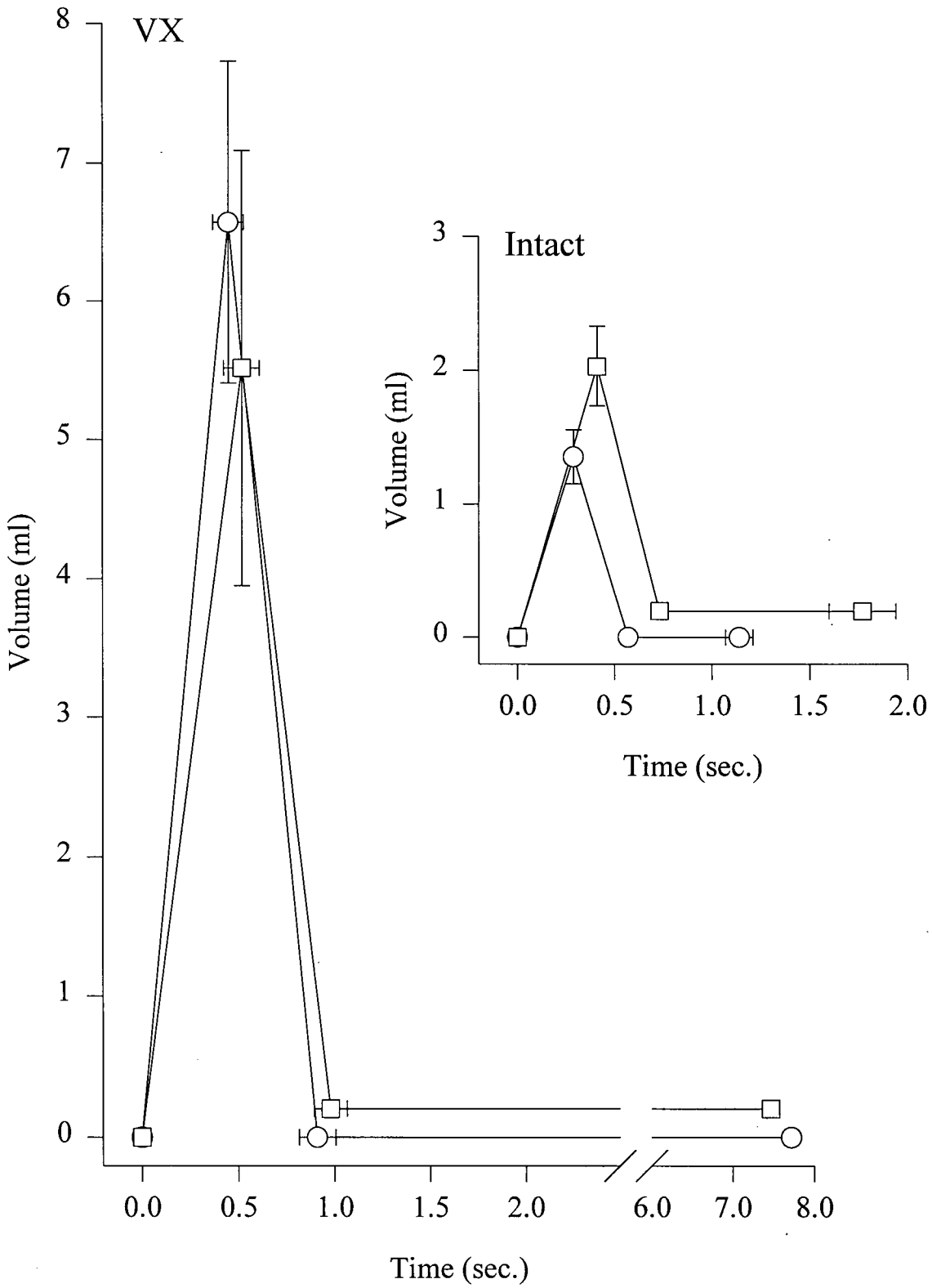

State I
State III
EEG
Ventilation
EEG
Ventilation
75 µv
2 ml
2 sec.

Fig. 3.5

Representation of mean (± standard error) inspiratory volumes and durations of inspiration (TI) and expiration (TEA and TEP) of anesthetized animals during State-I (○) and State-III (□), before (Intact) and after (VX) vagotomy. Statistical differences are noted in the text.


VX
Volume (ml)
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
6.0
7.0
8.0
Time (sec.)
Intact
Volume (ml)
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Time (sec.)

Vagotomy altered breathing pattern by decreasing f_R while increasing V_T, resulting in a slight but non-significant decrease in ventilation compared with pre-vagotomy values (Fig. 3.2). Breath timing was also altered, through increases in T_I and T_{EP}. Following vagotomy there were no alterations in breathing associated with transitions between State I and State III (Fig. 3.5).

3.3.3. Hibernating animals

3.3.3.1. Activation state

The EEG of animals hibernating with a body temperature of approximately 7°C showed little discernable activity. The ECG was clearly evident in the EEG. Ventilation in these animals occurred in distinct multi-breath episodes separated by prolonged periods of apnea (Fig. 3.6).

3.3.3.2. Vagal blockade

Sham injections of saline had no discernable effect on ventilation. Vagal blockade produced no significant changes in overall f_R or V_T, but did produce a significant decrease in V_E (Fig. 3.7). This ventilatory decrease resulted, not from a change in individual breaths (Fig. 3.8), but from a significant change in overall breathing pattern. Following vagal blockade, animals produced breathing episodes as frequently but took fewer breaths in each episode. The instantaneous frequency of breaths in each episode was not changed. No further change in ventilation was noted following a second infusion of xylocaine into the vagal cuff.

Fig. 3.6

Representative recordings of EEG and breathing during hibernation before (top) and after (bottom) vagal block. Breathing records are the integrated output of the pneumotachograph with inspiration as an upward deflection and peak height proportional to tidal volume.

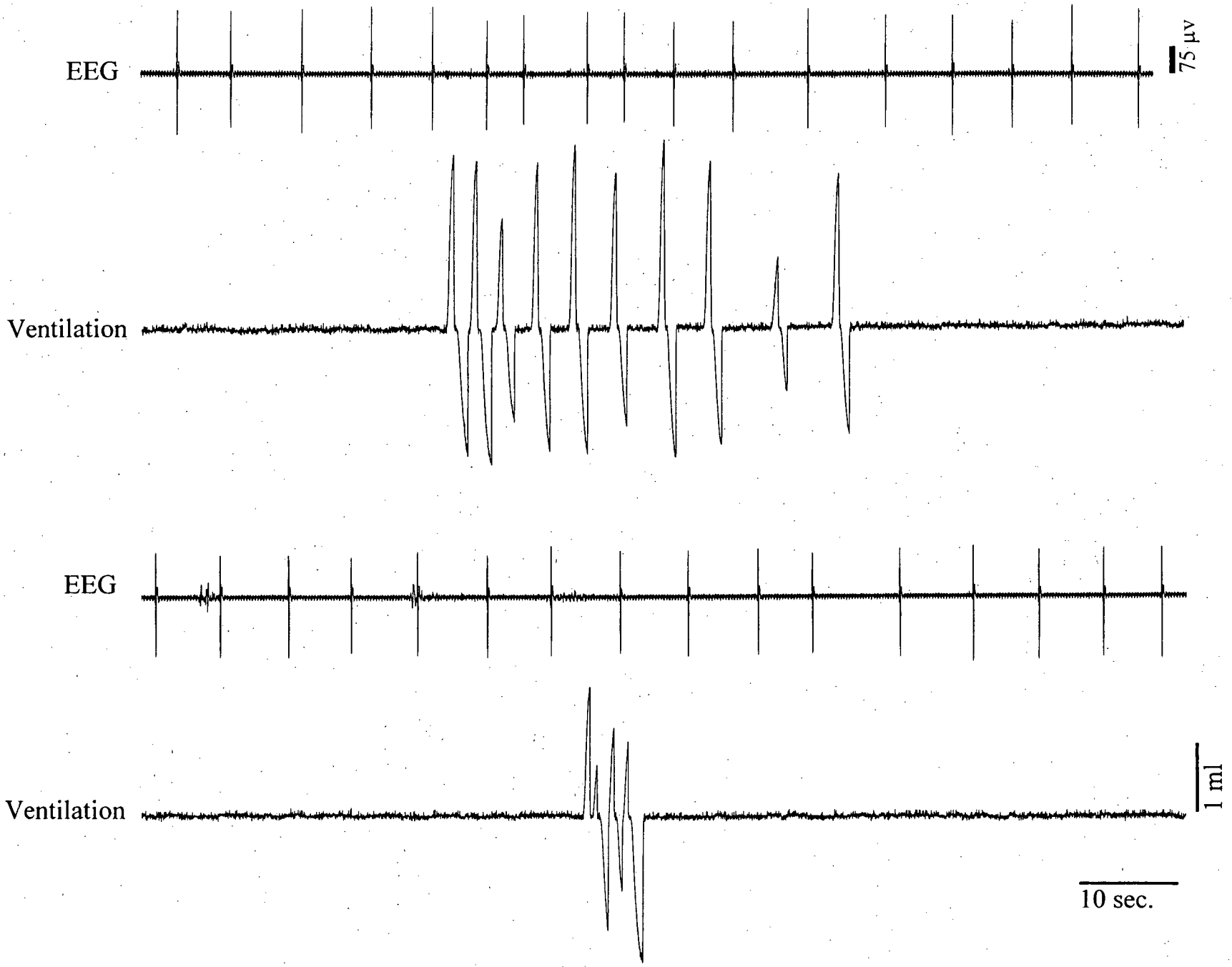

EEG
75 µv
Ventilation
EEG
Ventilation
1 ml
10 sec.

Fig 3.7

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and minute ventilation (V_E), breaths per episode, episodes per hour and the instantaneous frequency of breathing within episodes in hibernating animals before (open bars) and after (hatched bars) vagal block. The "*" denotes a significant difference from values in intact animals ($P<0.05$).

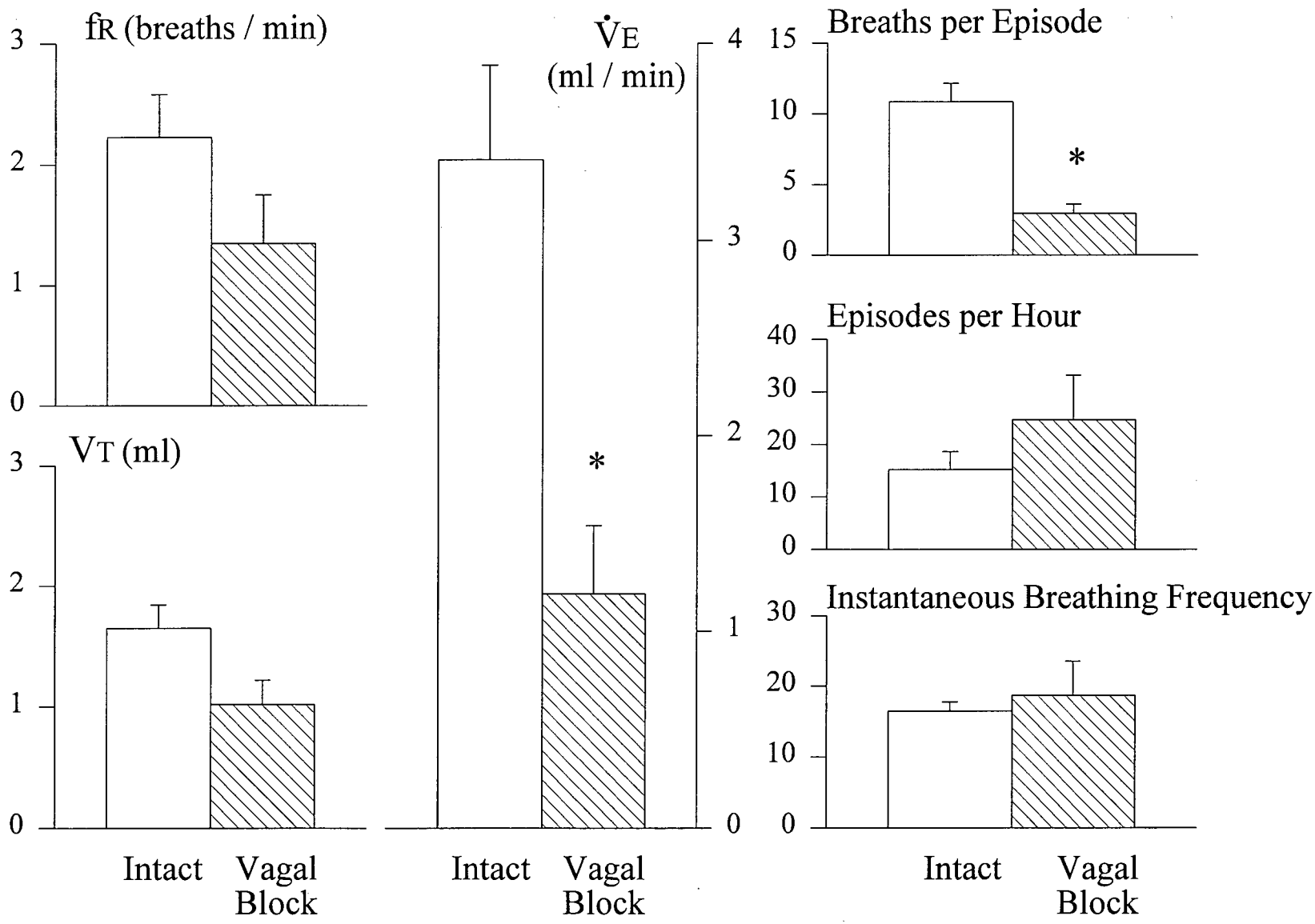
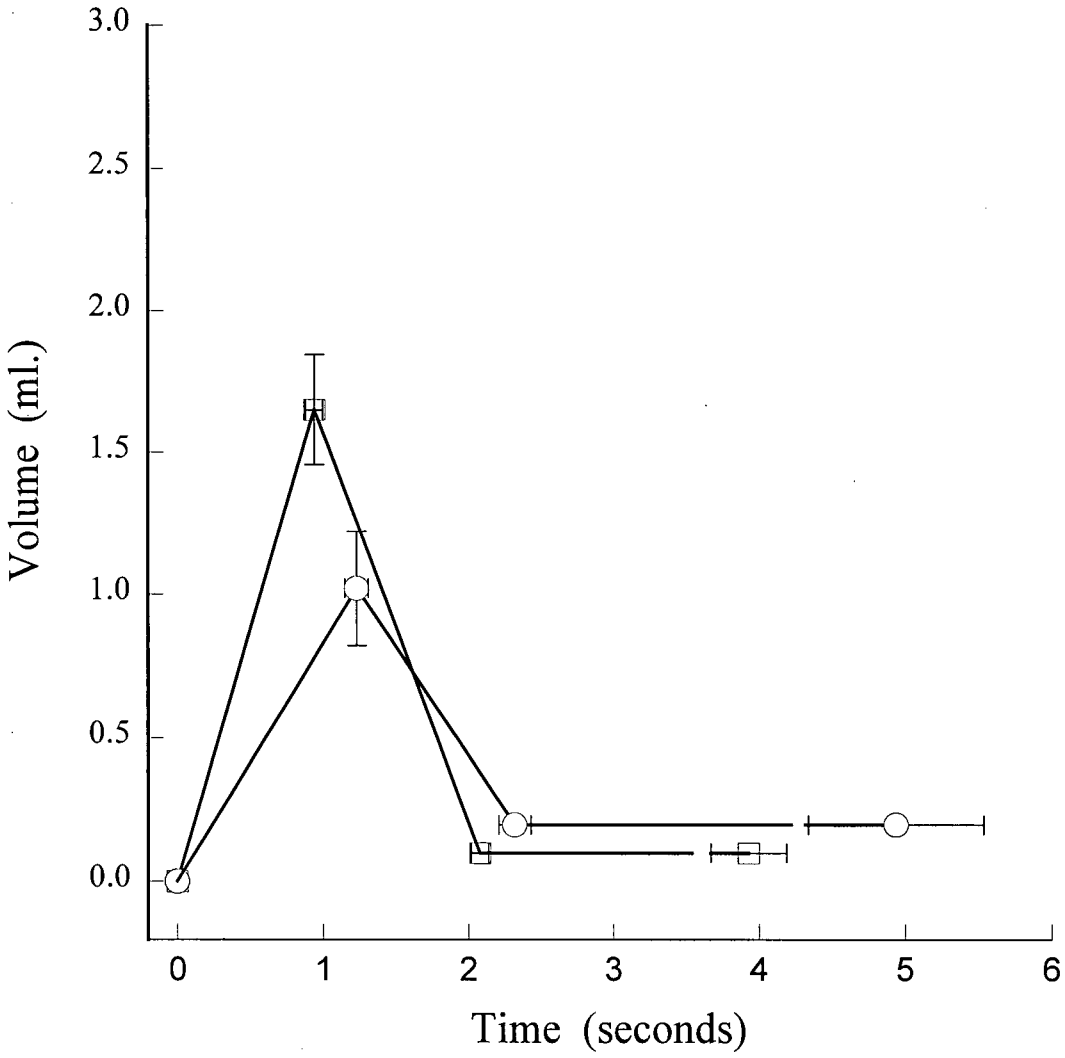

fR (breaths / min)
3
2
1
0
VT (ml)
3
2
1
0
Intact
Vagal Block
V̇E (ml / min)
4
3
2
1
0
*
Intact
Vagal Block
Breaths per Episode
15
10
5
0
*
Episodes per Hour
40
30
20
10
0
Instantaneous Breathing Frequency
30
20
10
0
Intact
Vagal Block

Fig. 3.8

Representation of mean (± standard error) inspiratory volumes and durations of inspiration (T_I) and expiration (T_{EA} and T_{EP}) of hibernating animals before (□) and after vagal block (○). Statistical differences are noted in the text.


3.0
2.5
2.0
1.5
1.0
0.5
0.0
Volume (ml.)
0
1
2
3
4
5
6
Time (seconds)

3.4. Discussion

3.4.1. Euthermic animals

3.4.1.1. Activation state

Breathing pattern was characterized during well established quiet-wakefulness (QW) and slow-wave sleep (SWS) and was significantly different between these states. During SWS, animals exhibited an increase in V_T and reduction in f_R compared to levels observed during QW (Fig. 3.2, 3). These results are consistent with the data of others demonstrating the influence of sleep and wakefulness on breathing in mammals (Hunter and Milsom, 1997; see Phillipson and Bowes, 1986 for review).

3.4.1.2. Vagal block

The effects of vagal blockade were immediate and extreme. Following the induction of vagal blockade, spontaneous ventilation was briefly replaced by periodic breaths involving large rapid expansions of the chest that appeared laboured and "gasp-like". In the first animal, the initiation of vagal blockade was followed by less than 5 seconds of relatively normal breathing and an unexpected apnea. The apnea was broken by three to four "gasp-like" breaths before artificial ventilation was initiated. In the next two animals the initiation of vagal blockade was followed by similar phenomena and artificial ventilation was begun within the initial apnea before the occurrence of the first "gasp-like" breath. In no case was spontaneous ventilation observed during 30 second periods where artificial ventilation was halted, during the period of vagal blockade. The second of these animals was revived by artificial ventilation approximately 30 minutes following the reversal of vagal blockade.

In the three subsequent experiments, artificial ventilation was initiated concurrently

with the induction of vagal blockade, before alterations in spontaneous breathing occurred. Again, however, spontaneous ventilation did not occur when artificial ventilation was interrupted during the period of vagal blockade. One of these animals was also revived but, again, only after 30 minutes of artificial ventilation following the reversal of vagal blockade.

There are three possible explanations for these results. The first is that vagal blockade resulted in upper airway obstruction which compromised ventilation. This is unlikely as the same results occurred in the single animals in which the glottis and upper airway were bi-passed. In addition, artificial ventilation was unencumbered in all cases. A second possibility is that ventilation was compromised by a lower airway obstruction due to pulmonary edema giving rise to hypoxemia and gasping. This is unlikely as there were no signs of fluid in the airway during artificial ventilation and no signs of tissue edema or fluid in the lung or airway at autopsy. The final possibility is that vagal input is necessary for eupnea. In its absence, eupnea fails leading to hypoxemia, gasping and death. These results support the conclusion that vagal feedback is essential for ventilation and that central apnea occurs in the absence of this feedback.

Adults of other rodents species, notably rats, do not exhibit the same catastrophic response to the removal of vagal feedback but exhibit the classical increases in tidal volume and decreases in breathing frequency (Martin-Body and Sinclair, 1987). Interestingly, however, vagotomy drastically compromises spontaneous ventilation in neonatal rats, resulting in profound reductions in breathing frequency and in prolonged apnea and respiratory arrest (Fedorko et al. 1988). One possible explanation of this stems from the relatively compliant chest wall of neonates (England and Strobel, 1997; England, pers. com.). Neonatal rats have a chest compliance which is greater than twice that of adults (Fisher and Mortola, 1980; Guslits

et al. 1987;). Having a compliant chest wall reduces the effort required for ventilation. This excess compliance, however, reduces chest elasticity and increases the risk of lung collapse . To prevent lung collapse during spontaneous ventilation, neonates must maintain a functional residual lung volume which is greater than the residual volume dictated by passive pulmonary mechanics. Thus, neonates, in contrast to adults, may be considerably more reliant on feedback from pulmonary stretch receptors to drive spontaneous ventilation; to the extent that ventilation fails in the absence of feedback from pulmonary stretch receptors. Interestingly, this failure results from central apnea in response to vagotomy and not from obstruction due to lung collapse (Fedorko et al. 1988). The chest compliance of golden-mantled ground squirrels is also twice that of adult rats (Milsom and Reid, 1995). This high compliance is believed to represent an adaptation for hibernation, allowing the animals to ventilate efficiently at low body temperatures. This high initial compliance opposes the physical tendency of the chest to stiffen as body temperature falls. Thus, while squirrels hibernating at 7°C exhibit a chest compliance which is half that of animals at 37°C, it is still equal to that of rats at 37°C (Milsom and Reid, 1995). The chest compliance of neonatal squirrels is unknown. It may be, however, that adult squirrels have retained a compliant chest throughout development to allow efficient ventilation during hibernation, but, as in the case of neonatal rats, when pulmonary receptor feedback is removed, spontaneous ventilation is abolished because the central respiratory control mechanism depends on activation by this feedback.

3.4.2. Anesthetized animals

3.4.2.1. Activation state

Rodents, anesthetized with urethane, have been previously shown to exhibit EEG

patterns similar to those observed during unanesthetized wakefulness and slow-wave sleep (Grahn et al. 1989). Based on similar observations, Hunter and Milsom (1997) hypothesized that activation states similar to normal sleep and wakefulness persist during urethane anesthesia and that the effects of these states on the respiratory control system of this species are analogous to the respiratory effects of wakefulness and slow-wave sleep. This does not imply that the mechanisms which produce States I and III of Urethane anesthesia are necessarily analogous to those which underlie wakefulness and sleep, rather that the effects of these states on respiratory control are similar.

3.4.2.2. Ventilation

In the present investigation, the fluctuations in ventilation between State-I and State-III appeared to be similar to those observed between quiet wakefulness and SWS (Fig. 3.2), as has been shown by others (Hunter and Milsom, 1997). Breathing was even and regular in State-I but became significantly slower with a noticeable, yet non-significant, increase in V_T in State-III ($p = 0.35$, by Students T-test). Inspiration, the end-inspiratory pause between breaths, and the overall respiratory cycle, all were longer in State-III than in State-I. T_{EA}, however, did not change between states (Fig. 3.5).

3.4.2.3. Vagotomy

Following vagotomy animals still cycled between States I and III. Vagotomy had no effect on overall ventilation, but did alter breathing pattern by increasing the duration of inspiration, elevating V_T, and increasing the duration of the end-expiratory pause between breaths resulting in a concurrent decrease in f_R (Fig. 3.2, 5). These data are consistent with the

role traditionally proposed for vagal feedback in ventilatory control (Cross et al. 1980; Coleridge and Coleridge, 1986 q.v.).

The vagus delivers phasic information pertaining to the stretch of the lung during inspiration, and tonic information concerning end expiratory volume or functional residual capacity of the lung. Phasic feedback acts to terminate inspiration via the Breuer-Hering reflex. In the absence of this feedback inspiration is prolonged and a larger inspiratory volume is achieved before other mechanisms act to terminate inspiration. If phasic feedback is removed, by the prevention of inspiratory flow, the ventilatory cycle is prolonged and breathing frequency falls, but this is due to a prolongation of the inspiratory phase rather than a prolongation of expiration (Feldman and Gautier, 1976; Cross et al 1980). Thus, the observation of breathing pattern change following vagotomy can not be explained simply as a loss of phasic vagal feedback, prompting the suggestion that tonic feedback may contribute to this response. Experiments which alter tonic and phasic vagal feedback independently, however, indicate that increases in tonic feedback prolong expiration and produce decreases in f_R, while decreases shorten expiration and increase breathing frequency (Knox, 1973; Trippenbach et al. 1985). It appears, therefore, that the effects of vagotomy cannot be attributed simply to reductions in either phasic or tonic vagal feedback. Thus, it is not clear what aspect of vagal feedback accounts for the prolongation of the end-expiratory pause and decrease in breathing frequency observed following vagotomy.

A conundrum remains, however, in that spontaneous ventilation ceases following removal of vagal feedback in unanesthetized squirrels but persists in anesthetized animals. These results suggest there is a facilitation of ventilation during anesthesia which is not otherwise present, or rather that there is a source of net inhibition of ventilation that is removed

during anesthesia. One highly speculative possibility is that there is a descending influence from a higher neural center which provides tonic negative modulation to the central respiratory controller. This negative modulation is countered by excitation provided by vagal feedback. In unanesthetized animals, removal of vagal feedback allows the negative modulation to be manifest, resulting in respiratory arrest. Anesthesia removes the influences of higher neural centers preferentially before affecting lower centers. During anesthesia, the source of such a descending negative modulation may itself be inhibited. In the absence of this tonic negative modulation, removal of the excitation provided by vagal feedback would simply allow the central respiratory controller to express its natural activity and ventilation would persist. If this were the case, one might expect anesthesia itself to facilitate ventilation, resulting from disinhibition, as the theoretical descending negative modulation was removed while excitation from vagal feedback persisted. Anesthesia, however, indirectly inhibits ventilation by influencing many other factors which could explain why such facilitation does not occur.

Following vagotomy, breathing no longer changed as animals cycled between State I and State III (Fig. 3.2, 5). This observation contrasts with data from humans, cats and dogs where fluctuations in breathing associated with transitions from wakefulness to sleep persist in the absence of pulmonary receptor feedback (Shea et al. 1988; Netick et al. 1980; Phillipson and Bowes 1986 q.v.). The similarities between the respiratory effects of changing arousal state and of vagotomy, and the absence of an influence of changes in state following vagotomy in squirrels, suggest either that changes in breathing with state result from altered integration of vagal input, or that the relative importance of vagal feedback is such that the transformation in ventilation associated with vagotomy exceeds and overshadows the more subtle changes associated with alterations in arousal state. That is, following vagotomy, f_R could not be

reduced further nor V_T increased further by changes in state.

3.4.3. Hibernating animals

3.4.3.1. Activation state

During hibernation EEG activity was diminished beyond a level that we could classify. This observation is similar to previous observations which document decreases in measurable forebrain activity during entrance into hibernation and during hibernation at different ambient and body temperatures (Walker et al. 1977).

3.4.3.2. Vagal blockade

Vagal blockade did not influence the shaping of individual breaths in hibernation (Fig. 3.8). This treatment, however, did reduce total ventilation and altered breathing pattern by producing fewer breaths within each breathing episode (Fig. 3.7). These data indicate that, in hibernation, vagal feedback acts to stimulate breathing by increasing the number of breaths that are taken each time breathing occurs.

In euthermic animals, phasic activity from pulmonary stretch receptors normally acts to terminate inspiration. As vagal blockade did not influence the timing component of individual breaths during hibernation, it may be concluded that this phasic activity is no longer involved in terminating inspiration in hibernation. Some aspect of feedback from pulmonary receptors in euthermic animals acts to stimulate breathing, as vagotomy results in a decreases in breathing frequency. Vagal blockade in hibernation resulted in fewer breaths per episode, indicating that some aspect of vagal feedback still has a significant influence, acting to promote or stimulate breathing when breathing occurs.

Lyman (1982) suggested that the effects of vagal cardiac efferent activity are selectively lost during hibernation, due to the inability of relatively thin parasympathetic nerves to conduct action potentials at these reduced temperatures. Recently, however, I have demonstrated that vagal output is still involved in cardio-respiratory coordination and producing respiratory-related sinus arrhythmia in deep hibernation (Harris and Milsom, 1995). The vagal efferent activity is simply reduced due to non-linear temperature (Q_{10}) effects. Data from the present study suggest that vagal afferent activity is also greatly reduced. The influence of phasic activity is no longer significant as loss of this phasic activity does not alter the individual breaths. Some aspect of vagal feedback is, however, present during hibernation as vagotomy does alter breathing pattern. This could reflect differential temperature effects on the phasic and tonic components of vagal afferent activity at any number of steps in the reflex pathway.

Again there is the conundrum of why spontaneous ventilation ceases following removal of vagal feedback in unanesthetized euthermic squirrels but persists during hibernation. The explanation may be similar to that proposed in the previous section. As animals enter hibernation, the metabolic activity of higher neural centers may be reduced to a greater degree than that of lower centers (Kilduff et al. 1982). If vagal feedback in euthermic animals is opposing a strong negative modulation that arises from a higher neural center, this center may be deactivated during hibernation resulting in a loss of the source of negative modulation. The vagal feedback is still facilitatory to breathing, yet this influence declines with temperature. During deep hibernation all that remains is the vagal stimulation of breathing which, when removed, results in the decline in the number of breaths occurring in the breathing episodes.

3.5. Conclusion

The data from euthermic animals indicate that spontaneous ventilation will not occur in the absence of vagal feedback. The reasons for this are enigmatic. Breathing does still occur after vagotomy in anesthetized animals and the data indicate that phasic and tonic vagal feedback then limit the volume of individual breaths and enhance breathing frequency. These observations contrast with the data obtained from hibernating animals. During hibernation vagal feedback does not seem to influence the characteristics of individual breaths suggesting that the phasic component of pulmonary receptor feedback is unimportant during hibernation. Tonic vagal feedback, however, retains an important role in the patterning of breathing episodes. How the relative role of vagal feedback is transformed under anesthetized and hibernating conditions remains unknown.

Chapter 4

The Influence of Vagal Feedback on the Ventilatory Chemoresponses of Ground Squirrels

Abstract

The influence of vagal afferent feedback on ventilatory responses to hypoxia and hypercapnia were assessed in the golden-mantled ground squirrel, *Spermophilis lateralis,* during, anesthesia and natural hibernation. Anesthetized animals cycled regularly between states of cortical activity similar to sleep and wakefulness. Hypoxia increased ventilation primarily through increases in f_R while hypercapnia increased ventilation primarily through increases in V_T in both states just as they did in unanesthetized animals. The hypercapnic ventilatory response was greater in the wake-like state than the sleep-like state in anesthetized animals whereas, in this study, there were no differences in the magnitude of hypoxic or hypercapnic ventilatory responses between wake and sleep in unanesthetized animals. Vagotomy altered breathing pattern in anesthetized animals by increasing V_T and decreasing f_R, depressed ventilatory responses to both hypoxia and hypercapnia, and eliminated the differences in ventilatory responses between states. Hibernating animals breathed episodically. They exhibited a robust ventilatory response to hypercapnia primarily due to an increase in the length of breathing episodes, but did not respond to hypoxia. Vagotomy depressed ventilation and abolished the hypercapnic ventilatory response. The data suggest that in the absence of vagal feedback, changes in breathing pattern compromise chemoreflexes.

4.1. Introduction

During hibernation, ground squirrels exhibit a curious ventilatory response to progressive hypercapnia. These animals exhibit a linear increase in ventilation as the fraction of inspired CO_2 is increased from 2 to approximately 8 %. As CO_2 levels are raised above this range, however, ventilation fails to increase further but is maintained at a "plateau" level until arousal from hibernation is initiated. At this point, ventilation again increases (Harris and Milsom, 1994). This response is unlike that of euthermic ground squirrels or of other animals, all of which generally exhibit a linear increase in ventilation with progressive increases in the level of hypercapnia (see Cunningham et al. 1986 for review). Rabbits, however, also exhibit a ventilatory "plateau" in their hypercapnic response curve following vagotomy (Richardson and Widdicomb, 1969). The parallel between the shape of the hypercapnic ventilatory response curve in hibernating squirrels and vagotomized rabbits, and the contention that some vagally mediated reflexes are absent during hibernation (Lyman, 1982), calls into question the role of vagal afferent feedback in modulating ventilatory chemoresponses during hibernation.

In chapter 3, it was demonstrated that ventilation in euthermic golden-mantled ground squirrels was critically dependant on intact vagus nerves. These squirrels did not exhibit spontaneous ventilation in the absence of vagal feedback. In anesthetized animals, on the other hand, ventilation continued post-vagotomy, but the shape of individual breaths was altered. Breathing was slower and deeper indicating that while vagal feedback was no longer critical for breathing, it still played an important role in shaping breathing pattern. In hibernating animals, vagal feedback was even less critical, it increased the overall level of ventilation by increasing the length of breathing episodes, but did not influence the frequency or depth of individual breaths.

Given the results of this previous investigation it would be predicted that vagal feedback should at least influence the manner in which changes in blood gases alter the episodic breathing pattern in hibernating squirrels. We would also predict that vagal feedback would influence the effect of changes in blood gases on both breathing pattern and the magnitude of the ventilatory responses in anesthetized, euthermic animals, much as it does in other species. Vagal feedback should have an even stronger effect on chemoresponses in unanesthetized euthermic animals. This study was designed to test the first two predictions by comparing the ventilatory responses of squirrels to hypoxia and hypercapnia before and after vagotomy or vagal blockade. Given that vagotomy completely eliminated breathing in unanesthetized euthermic animals, however, this approach could not be used to test the last prediction.

4.2. Methods

4.2.1. Instrumentation

Experiments were performed on twenty five of the same adult golden-mantled ground squirrels *(Spermophilus lateralis)* described in chapter 3. Animals used in the chronic studies were surgically instrumented *a priori*. Animals were fitted with a vagal infusion cuff, four electroencephalographic (EEG), two electro cardiographic (ECG), two electromyographic (EMG) and two respiratory impedance electrodes, and the contralateral vagus to the one receiving the infusion cuff was sectioned. All procedures were performed as outlined in the general methods section (chapter 2).

4.2.2. Euthermic animals (chronic study)

Six squirrels were selected for this portion of the study. For each experiment, the animals were transferred to a whole body plethysmograph supplied with air and monitored for at least 2 hours. They were then randomly exposed to hypercapnic (5% CO_2 in air) and hypoxic (10% O_2 in nitrogen) gas mixtures for at least 30 minute periods separated by 30 minutes of exposure to air. Since unanesthetized animals do not exhibit spontaneous breathing following vagal blockade (chapter 3), hypoxic and hypercapnic ventilatory responses could only be characterized in intact animals in this group.

4.2.3. Hibernating animals (chronic study)

Twelve animals selected for this portion of the study underwent surgery during the winter. Following recovery, these animals reentered hibernation. During deep hibernation, animals were transferred to a plexiglass box (12 x 20 x 10 cm) within a laboratory controlled-

environment chamber. Each box was supplied with air at a flow rate of 500 ml per minute. Ambient temperature was held at 5 ± 1 °C. Animals were fitted with a mask connected to a resistance pneumotachograph to measure ventilation. The cannula to the vagal cuff was connected to a 3 cc. syringe via a length of polyethylene tubing (PE 50). The syringe and tubing were filled with xylocaine except for the final 0.2 ml at the tip which was filled with saline, separated from the xylocaine by a small air bubble.

Animals were monitored; i) for at least two hours to provide initial control values during exposure to air, ii) for at least two hours while the test box was supplied with hypercapnic (5% CO_2 in air) gas, iii) for at least two hours while the test box was supplied with hypoxic (7% O_2 in N_2) gas, iv) for at least one hour following a 0.2 ml infusions of saline (sham treatment) into the vagal cuff, v) for at least two hours following a 0.2 to 0.4 ml injection of xylocaine (vagal blockade) into the vagal cuff, and vi) again during 2 hour periods of exposure to hypercapnic and hypoxic gases, and finally, vii) for at least one hour following an additional 0.2 ml injection of xylocaine into the vagal cuff.

4.2.4. Anesthetized animals (acute study)

Seven ground squirrels were used in this series of experiments. Animals were anesthetized with urethane, tracheotomized, fitted with EEG, ECG, EMG and respiratory impedance electrodes and had their cervical vagi isolated, as described in Chapter 2.

Animals were supplied with air and monitored for at least 2 hours, and then randomly exposed to hypercapnic (5% CO_2 in air) and hypoxic (10% O_2 in nitrogen) gas mixtures for at least 30 minute periods separated by 30 minutes of exposure to air. Following hypoxic and hypercapnic treatment, animals were returned to air and underwent bilateral cervical vagotomy.

Vagotomy was achieved by injecting 0.2 ml of xylocaine into the previously implanted polyethelyne sleeves, and then by severing the nerves by pulling the loop of suture through each sleeve. At least 30 minutes after vagotomy, animals were again exposed to hypoxic and hypercapnic gas mixtures.

Squirrels anesthetized with Urethane cycle through states of cortical activity similar to wakefulness (State I) and slow-wave sleep (State III) (Grahn, et al. 1989; Hunter et al. 1997; chapter 3). In initial experiments, following vagotomy the State-III activation state was not seen during exposure to these levels of hypoxia and hypercapnia, and in some instances exposure to these gases produced an epileptiform EEG pattern suggestive of ineffective ventilation. As a consequence, in this series of experiments, ventilation was also characterized before vagotomy during exposure to an additional intermediate level of hypoxia (30 minutes of 12% O_2 followed by the 30 minutes of 5% O_2) and hypercapnia (30 minutes of 3.5% CO_2 followed by the 30 minutes of 5% CO_2), and after vagotomy, only during exposure to 12% O_2 and 3.5% CO_2.

4.3. Results

4.3.1 Euthermic animals

Ventilation was analyzed only during well established periods of quiet wakefulness (QW) and slow-wave sleep (SWS) (Fig. 4.1). During QW, hypoxia produced non-significant increases in both respiratory frequency (f_R) (from 112.0 ± 8.0 to 159.1 ± 29.6 br/min) and tidal volume (V_T) (from 2.6 ± 0.4 to 4.8 ± 1.1 ml), which together resulted in a significant increase in ventilation (V_E)(153%, from 276.1 ± 38.6 to 698.3 ± 155.9 ml/min) (Fig. 4.2). There were no significant changes in the timing components of each breath (Fig. 4.3). Hypercapnia produced a 63% decrease in f_R (from 112.0 ± 8.0 to 42.1 ± 9.5) and a 342% increase in V_T (from 2.6 ± 0.4 to 11.5 ± 1.7), resulting in a 63% increase in V_E (from 276.1 ± 38.6 to 450.0 ± 87.1) (Fig. 4.2). Decreases in f_R resulted from increases in the duration of inspiration (T_I) the duration of end-expiratory pauses (T_{EP}), while increases in V_T resulted from an increase in inspiratory duration (T_I) with no change in mean inspiratory flow rate or the duration of expiratory flow (T_{EA}) (Fig. 4.3).

During SWS, hypoxia produced a 197% increase in f_R (from 46.4 ± 4.3 to 138.1 ± 27.0) and a non-significant decrease in V_T (from 5.4 ± 0.7 to 4.6 ± 1.4), resulting in an 140% increase in V_E (from 247.7 ± 25.8 to 595.0 ± 181.2) (Fig. 4.2). These increases in frequency resulted from significant reductions in both T_I and T_{EP} (Fig. 4.3). Hypercapnia produced a non-significant decrease in f_R (from 46.4 ± 4.3 to 38.7 ± 9.6) and a 122% increase in V_T (from 5.4 ± 0.7 to 12.0 ± 1.5), resulting in an 102% increase in V_E (from 247.7 ± 25.8 to 500.1 ± 180.4) (Fig. 4.2). In SWS, T_I was not significantly altered, and increases in V_T resulted from increases in mean inspiratory flow rate (Fig. 4.3).

Fig. 4.1

Representative recordings of EEG and breathing of a squirrel exposed to A) air, B) hypoxia (10% O_2) and C) hypercapnia (5% CO_2) during quiet wakefulness (QW) and slow-wave sleep (SWS). Breathing records are from the output of the plethysmograph, with inspiration as an upward deflection and tidal volume proportional to the area under the deflection.

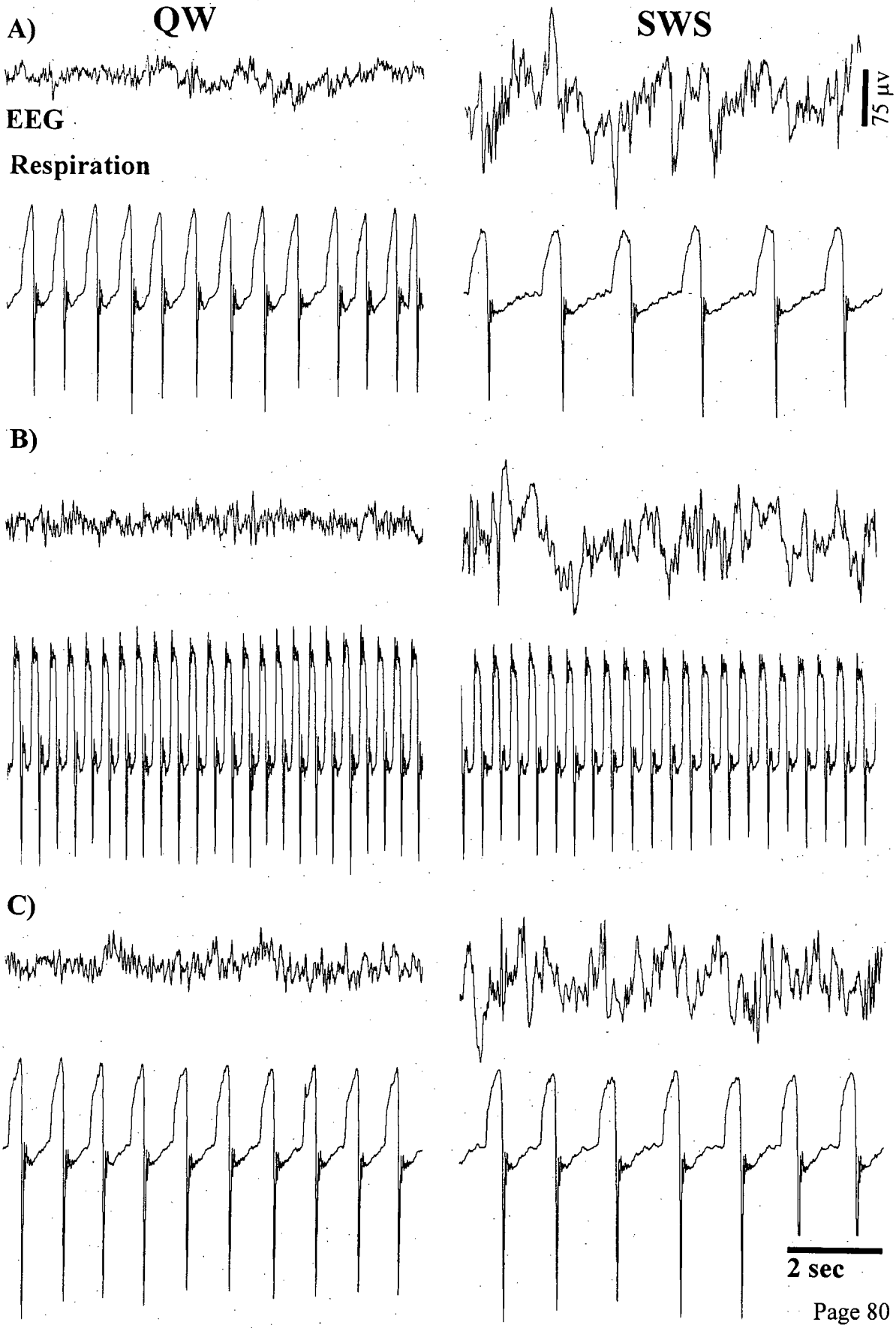

A)
QW
SWS
EEG
Respiration
75 µv
B)
C)
2 sec

Fig. 4.2

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) during exposure to air, 10% O_2 and 5% CO_2 in quiet wake (QW, open bars) and slow-wave sleep (SWS, hatched bars). The "*" denotes a significant difference from the QW value within the gas treatment, while the "#" denotes a significant difference from the same state during exposure to air ($P<0.05$).

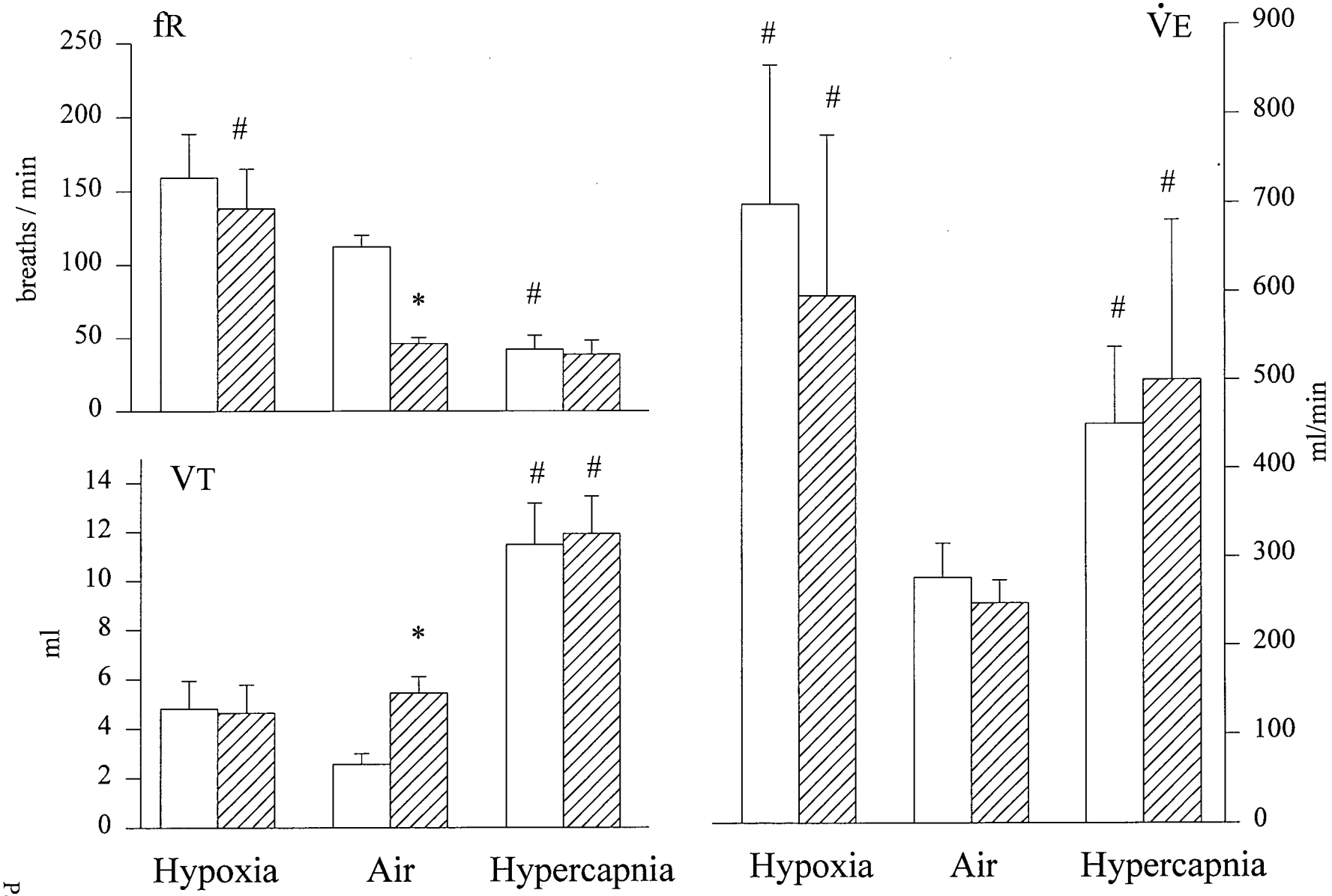
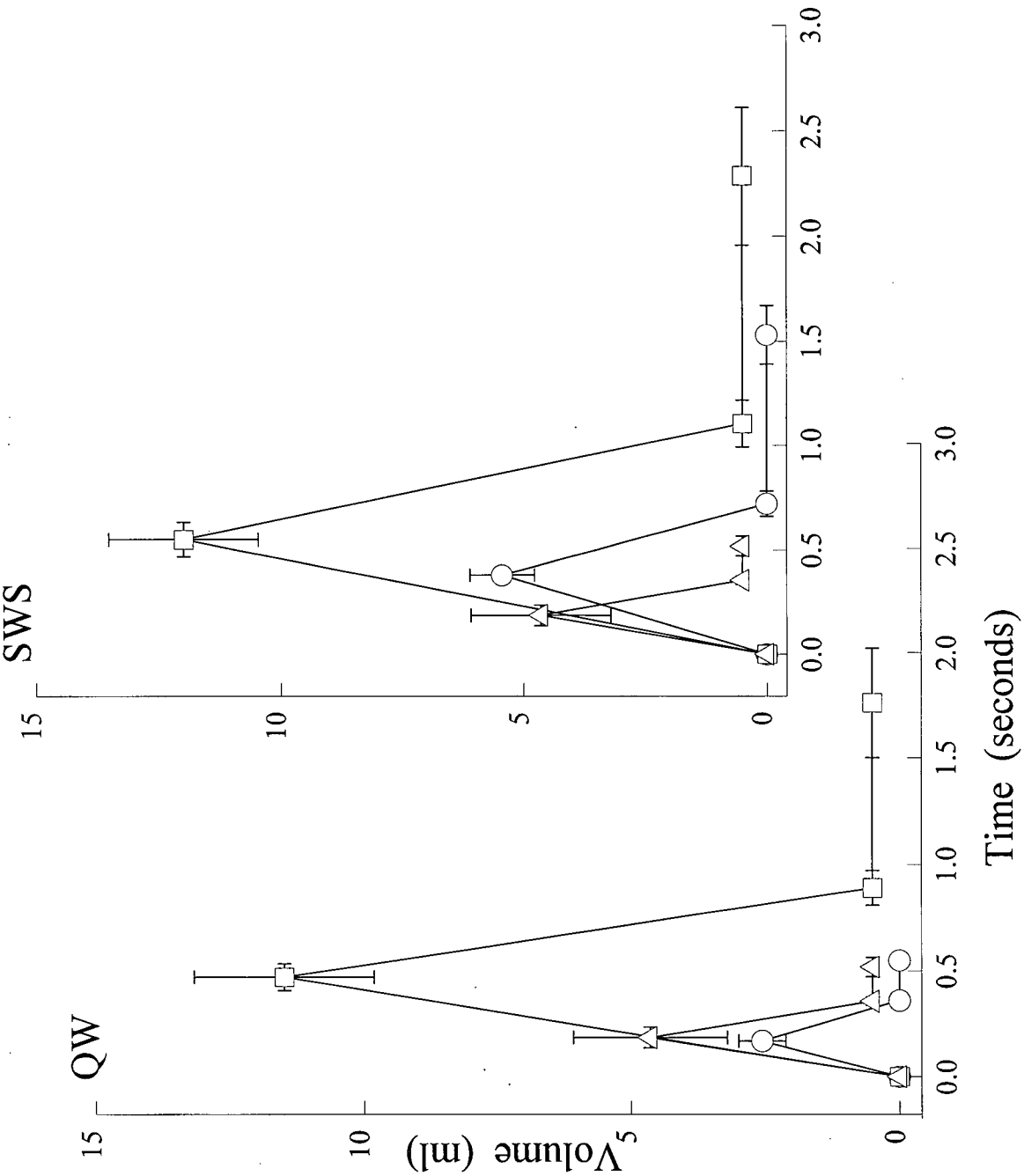

fR
breaths / min
250
200
150
100
50
0
#
*
#
VT
ml
14
12
10
8
6
4
2
0
#
#
*
Hypoxia
Air
Hypercapnia
V̇E
ml/min
900
800
700
600
500
400
300
200
100
0
#
#
#
Hypoxia
Air
Hypercapnia

Fig. 4.3

Representation of mean (± standard error) inspiratory volumes and durations of inspiration (T_I) and the components of expiration (T_{EA} and T_{EP}) in unanesthetized animals during exposure to air (○), 10% O_2 (△) and 5% CO_2 (□) in quiet wake (QW) and slow-wave sleep (SWS). Statistical differences are noted in the text. Note that in different plots the end-expiratory volumes have been offset from zero for clarity.


QW
SWS
Volume (ml)
Time (seconds)
0
5
10
15
0.0
0.5
1.0
1.5
2.0
2.5
3.0

4.3.2 Anesthetized animals

4.3.2.1. Intact

Under Urethane anesthesia, animals exhibited distinct "awake-like" and "slow-wave sleep-like" patterns of cortical activity (States I and III respectively; Hunter and Milsom, 1997; chapter 3) (Fig. 4.4). During State I, hypoxia produced a 113% increase in f_R (from 56.4 ± 4.7 to 119.9 ± 7.8 br/min) and a non-significant decrease in V_T (from 1.33 ± 0.2 to 1.03 ± 0.14 ml), resulting in a 54% increase in $\dot{V}_E$ (from 71.4 ± 9.0 to 109.8 ± 23.0 ml/min) (Fig. 4.5). Breathing frequency increased due to a decrease in T_{EP} while changes in V_T resulted from an increase in inspiratory flow, as T_I was not altered (Fig. 4.6). Hypercapnia produced a 68% increase in f_R (from 56.4 ± 4.7 to 94.6 ± 24.7) and a 160% increase in V_T (from 1.33 ± 0.20 to 3.47 ± 0.52), resulting in a 333% increase in $\dot{V}_E$ (from 71.4 ± 9.0 to 309.5 ± 67.5) (Fig. 4.5). Breathing frequency was increased by a decrease in T_{EP} while T_I was unchanged (Fig. 4.6).

During State III, hypoxia produced a 180% increase in f_R (from 35.9 ± 4.2 to 100.3 ± 11.1) and a 44% decrease in V_T (from 1.94 ± 0.39 to 1.08 ± 0.17), resulting in an 43% increase in $\dot{V}_E$ (from 70.33 ± 16.23 to 100.65 ± 9.52) (Fig. 4.5). Breathing frequency increased due to decreases in both T_{EP} and T_I, while the decrease in V_T resulted from a constant inspiratory flow occurring over a reduced T_I (Fig. 4.6). Hypercapnia produced a non-significant increase in f_R (from 35.9 ± 4.2 to 39.1 ± 6.4) and a 96% increase in V_T (from 1.94 ± 0.39 to 3.80 ± 0.45), resulting in a 106% increase in $\dot{V}_E$ (from 70.33 ± 16.23 to 144.67 ± 20.75) (Fig. 4.5). Tidal volume changes resulted from an increase in inspiratory flow, as T_I was unchanged (Fig. 4.6).

Fig. 4.4

Representative recordings of EEG and breathing of an intact anesthetized squirrel exposed to A) air, B) hypoxia (10% O_2) and C) hypercapnia (5% CO_2) during State I and State III. Breathing records are the integrated output of the pneumotachograph with inspiration as an upward deflection and peak height proportional to tidal volume.

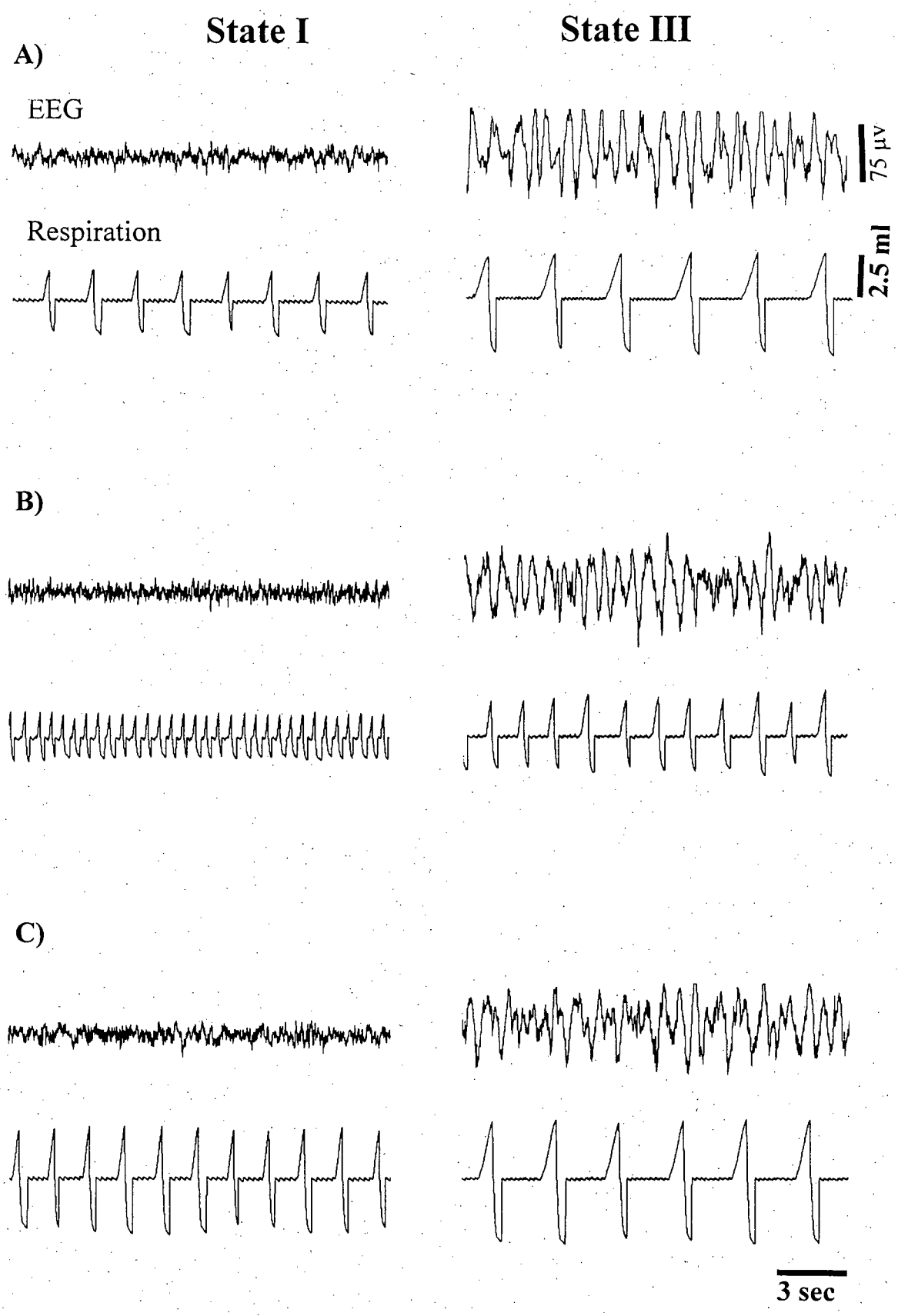

State I
State III
A)
EEG
Respiration
75 µv
2.5 ml
B)
C)
3 sec

Fig. 4.5

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) of intact anesthetized animals during exposure to air (open bars), 10% O_2 (hatched bars) and 5% CO_2 (cross-hatched bars) in States I and III. The "*" denotes a significant difference from the air value within the same state, while the "#" denotes a significant difference from the same state (I or III) in intact animals ($P<0.05$).

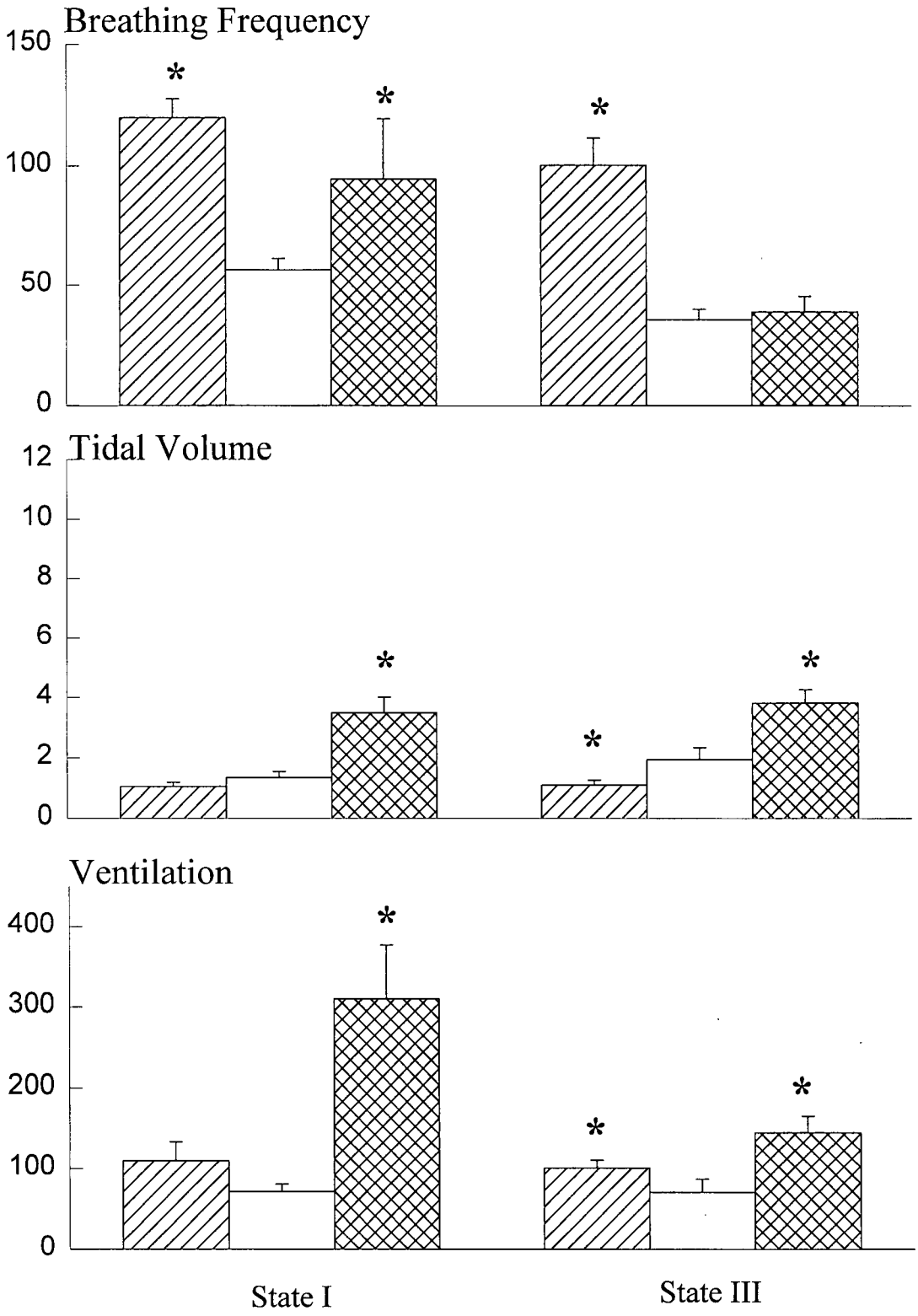
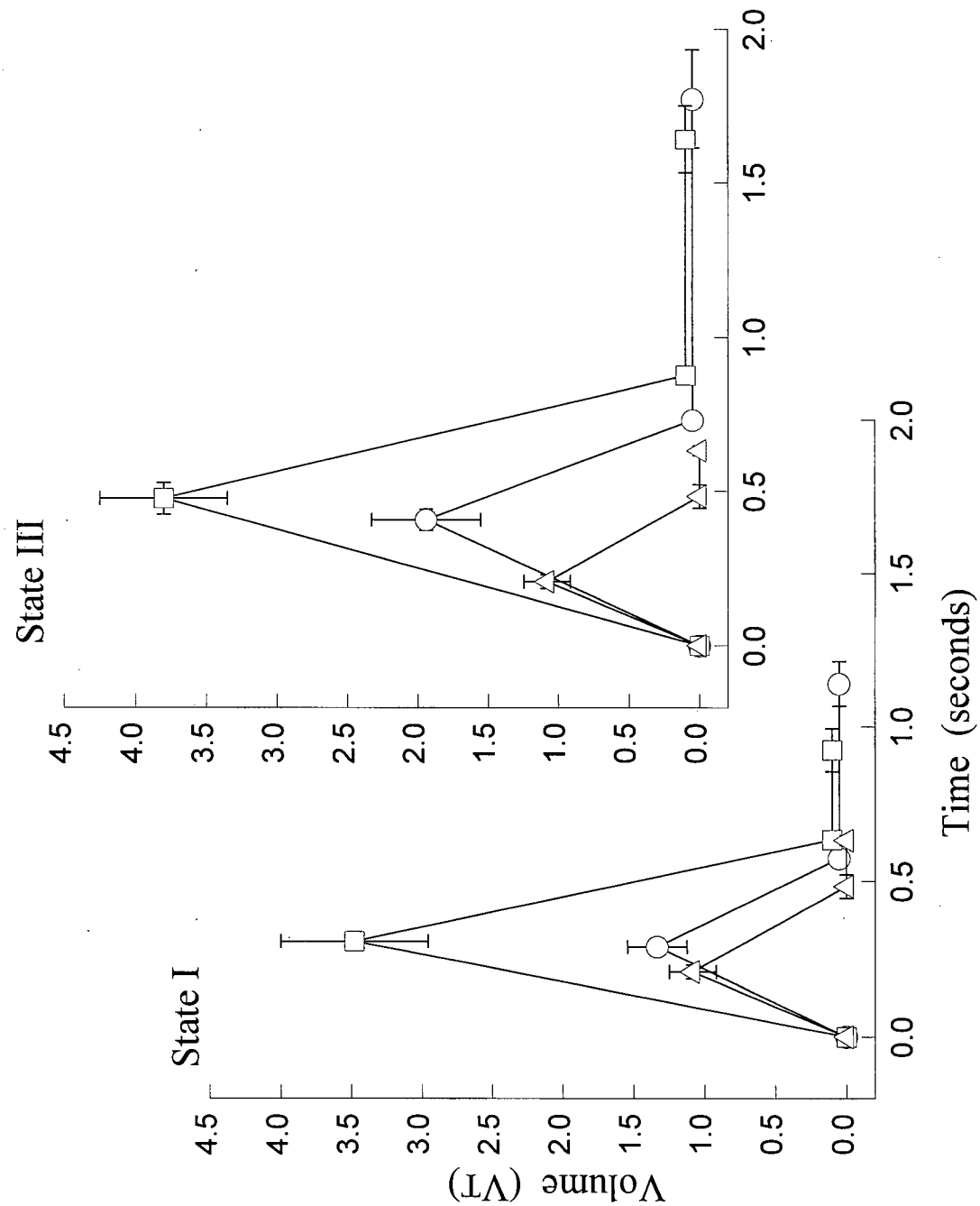

Breathing Frequency
150
100
50
0
Tidal Volume
12
10
8
6
4
2
0
Ventilation
400
300
200
100
0
State I
State III

Fig. 4.6

Representation of mean (± standard error) inspiratory volumes and durations of inspiration (T_I) and the components of expiration (T_{EA} and T_{EP}) in anesthetized animals during exposure to air (○), 10% O_2 (△) and 5% CO_2 (□), in States I and III. Statistical differences are noted in the text.


State III
State I
Volume (VT)
Time (seconds)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0

4.3.2.2. Post - vagotomy

Vagotomy altered breathing pattern during both State-I and State-III by decreasing f_R while increasing V_T, resulting in a slight but non-significant decrease in V_E (Fig. 4.7, 8,9). The alterations in breathing formerly associated with transitions between State I and State III no longer occurred following vagotomy (Fig. 4.7, 8, 9). Ventilation was stimulated by both hypoxia and hypercapnia following vagotomy. While neither hypoxia nor hypercapnia produced significant increases in f_R and V_T from the levels observed during air exposure, the combination of effects produced significant increases in V_E (Fig. 4.8). In State-I, hypoxia increased ventilation by 73 % (from 54.8 ± 8.3 to 94.9 ± 18.3 ml/min) while hypercapnia increased ventilation by 69 % (from 54.8 ± 8.3 to 92.4 ± 15.8 ml/min). In State-III, hypoxia and hypercapnia both produced marginally significant increases in ventilation (hypoxia: 92 %, from 40.3 ± 7.4 to 77.3 ± 14.0 ml/min, P=0.09; hypercapnia: 104 %, from 40.3 ± 7.4 to 82.3 ± 16.6 ml/min, P=0.075). The only significant alteration in breathing pattern following vagotomy was a decrease in T_{EP} associated with hypoxic exposure in State I (Fig. 4.9).

4.3.3. Hibernating animals

4.3.3.1. Intact

Resting ventilation in hibernating animals occurred in distinct multi-breath episodes separated by prolonged periods of apnea (Fig. 4.10). There were no significant changes in breathing pattern associated with exposure to hypoxia. Exposure to hypercapnia, however, increased ventilation by 370 % (from 3.4 ± 0.5 to 16.1 ± 3.8 ml/min), primarily due to a 285 % increase in f_R (from 2.2 ± 0.4 to 8.6 ± 1.2 br/min) (Fig. 4.11). Increases in breathing frequency resulted from changes in the length of breathing episodes, there was a 137 % increase in the

Fig. 4.7

Representative recordings of EEG and breathing of a vagotomized anesthetized squirrel exposed to A) air, B) hypoxia (12% O_2) and C) hypercapnia (3.5% CO_2) during State I and State III. Breathing records are the integrated output of the pneumotachograph with inspiration as an upward deflection and peak height proportional to tidal volume.

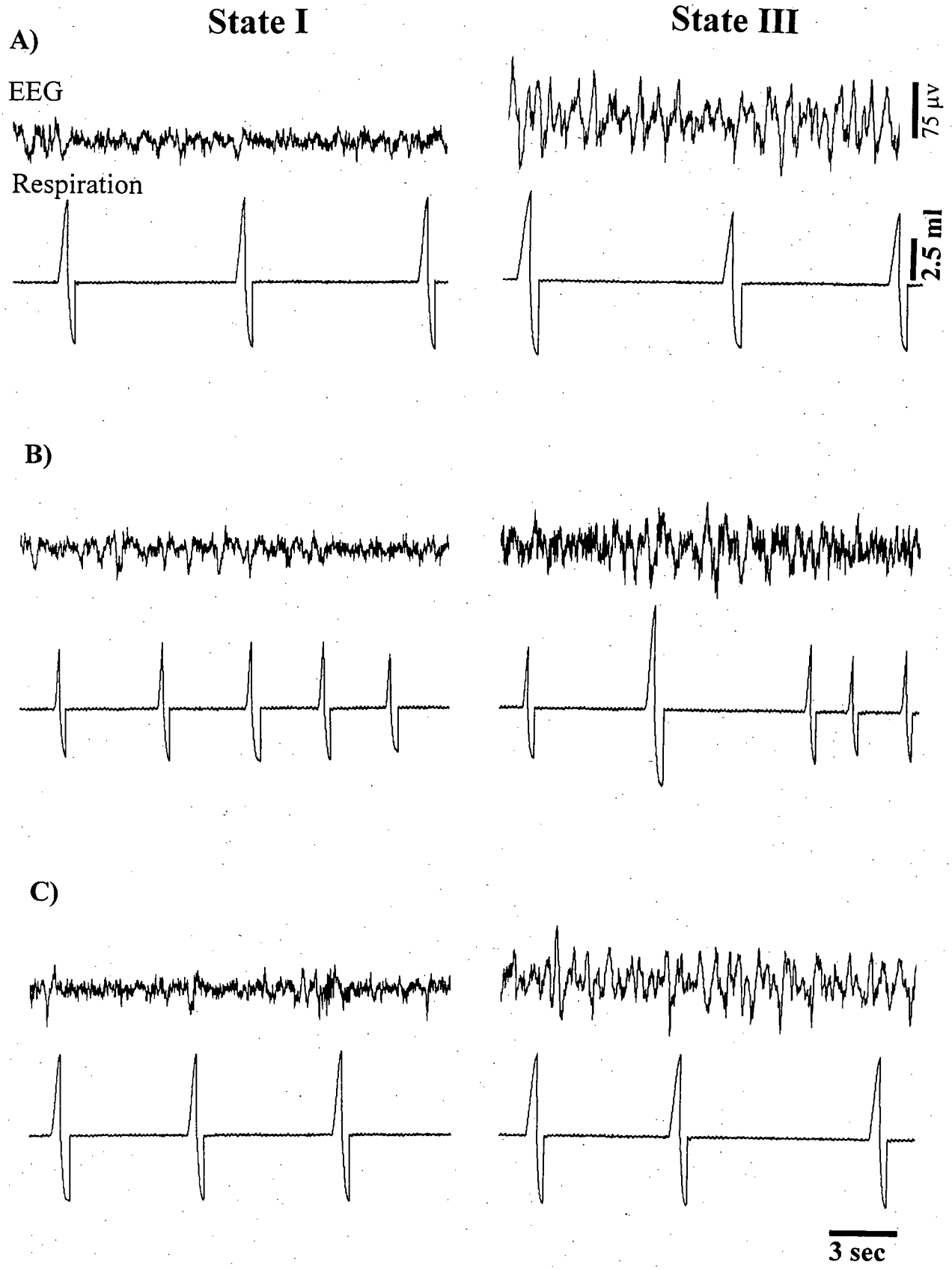

State I
State III
A)
EEG
Respiration
75 µv
2.5 ml
B)
C)
3 sec

Fig. 4.8

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) of vagotomized anesthetized animals during exposure to air (open bars), 12% O_2 (hatched bars) and 3.5% CO_2 (cross-hatched bars) in States I and III. The "*" denotes a significant difference from the air value within the same state, while the "#" denotes a significant difference from the same state (I or III) in intact animals ($P<0.05$). The "+" denotes a significant difference from the air value within the state at a $P<0.10$.

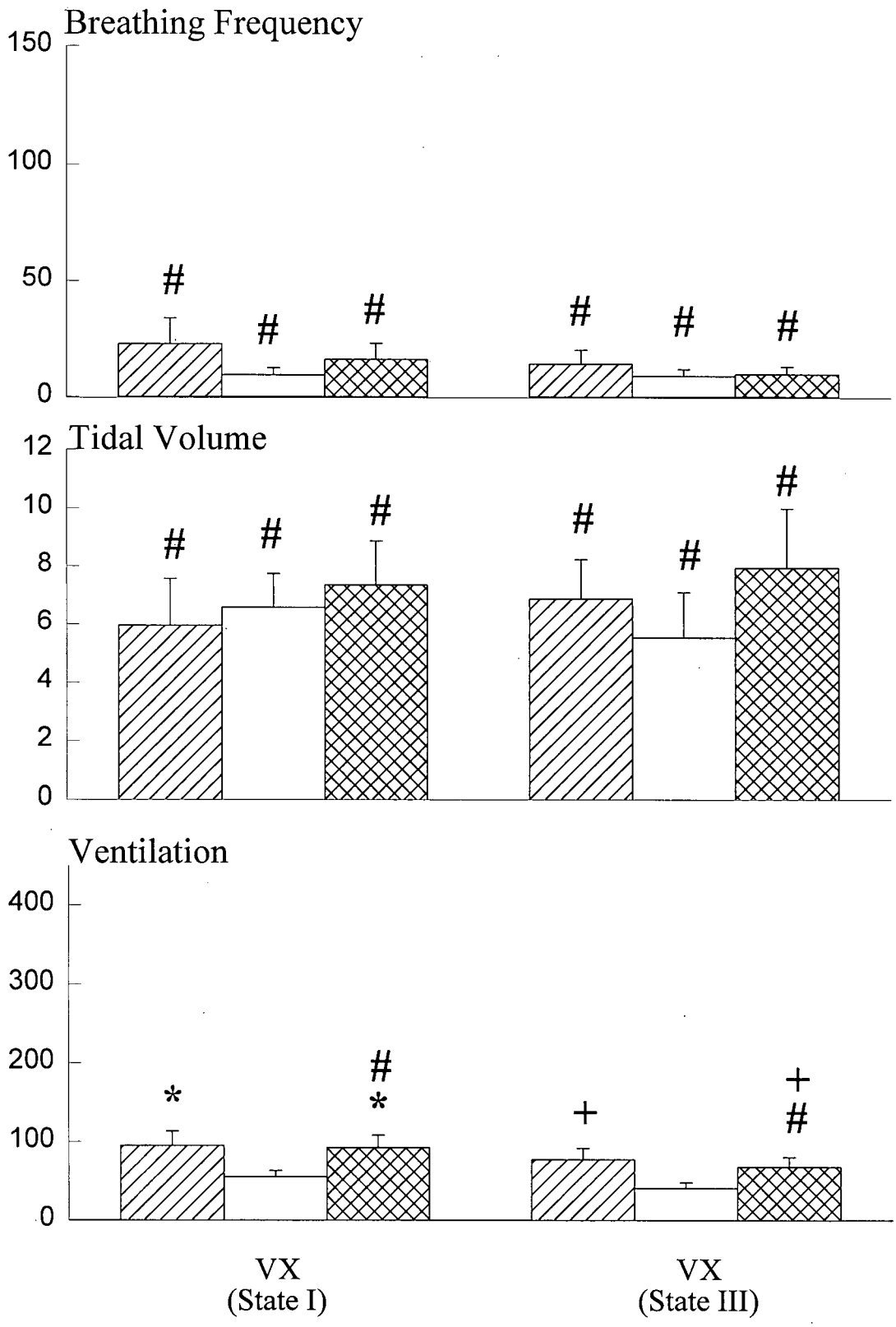
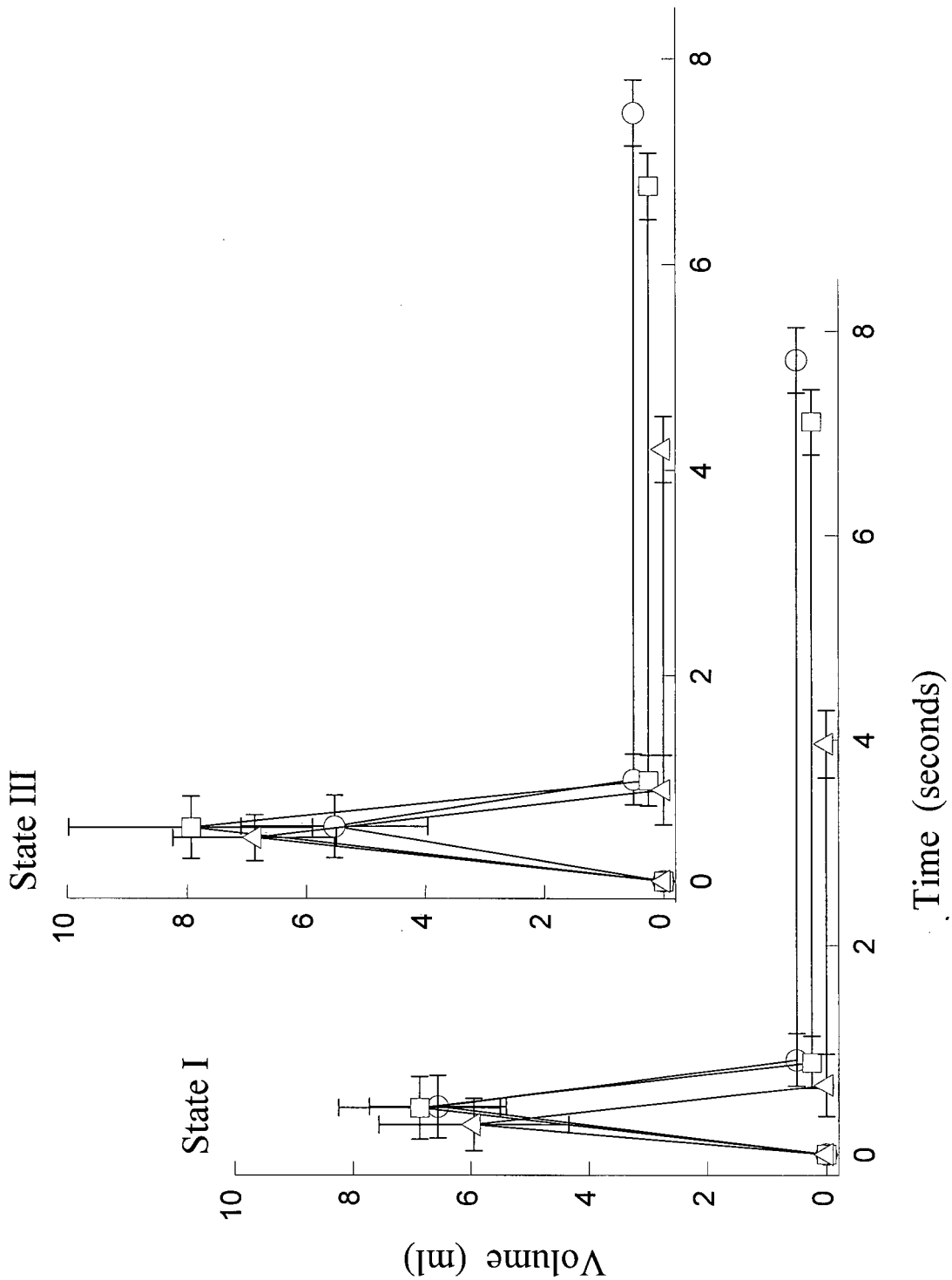

Breathing Frequency
150
100
50
0
#
#
#
#
#
#
Tidal Volume
12
10
8
6
4
2
0
#
#
#
#
#
#
Ventilation
400
300
200
100
0
*
#
*
+
+
#
VX
(State I)
VX
(State III)

Fig. 4.9

Representation of mean (± standard error) inspiratory volumes and durations of inspiration (T_I) and the components of expiration (T_{EA} and T_{EP}) in anesthetized animals in States I and III during exposure to air (○), 12% O_2 (△) and 3.5% CO_2 (□), after vagotomy. Statistical differences are noted in the text.


State III
State I
Volume (ml)
Time (seconds)
0
2
4
6
8
10

number of breaths per episode (from 10.9 ± 1.3 to 25.8 ± 4.8), while the frequency of episodes themselves was not significantly changed (Fig. 4.11). Tidal volume, the instantaneous frequency of breathing within each episode, and the pattern of individual breaths all were constant between gas treatments (Fig. 4.10,11, 12).

4.3.3.2. Post - vagal blockade

Sham injections of saline had no discernable influence on breathing while vagal blockade resulted in a 65 % decrease in $\dot{V}_E$ (from 3.4 ± 0.5 to 1.9 ± 0.4 ml/min) stemming from a 73 % decrease in the number of breaths per episode (from 10.9 ± 1.3 to 3.0 ± 0.7) and a non-significant fall in the size of each breath (Fig. 4.10, 11). There were no significant changes in breathing pattern associated with exposure to hypoxia. Hypercapnia following vagal blockade did not result in significant increases in ventilation, although non-significant increases in the frequency of episodes (154 % increase from 24.7 ± 8.5 to 62.8 ± 14.9 episodes/hr, P= 0.21 by Student's T-test) and number of breaths per episode (266 % increase from 3.0 ± 0.7 to 11.0 ± 7.16, P =0.39 by Student's T-test) were apparent. Again, tidal volume, instantaneous frequency of breathing within each episode, and the timing of individual breaths all remained constant between gas treatments and values were not different from those observed before vagal blockade (Fig. 4.11, 12).

Fig. 4.10

Representative recordings of breathing from a hibernating squirrel during exposure to A) air, B) hypoxia (7% O_2) and C) hypercapnia (5% CO_2), before (Intact) and after (Blocked) vagal blockade. Breathing records are the direct output of the pneumotachograph with inspiration as an upward deflection. Tidal volume is proportional to the area under the deflection.

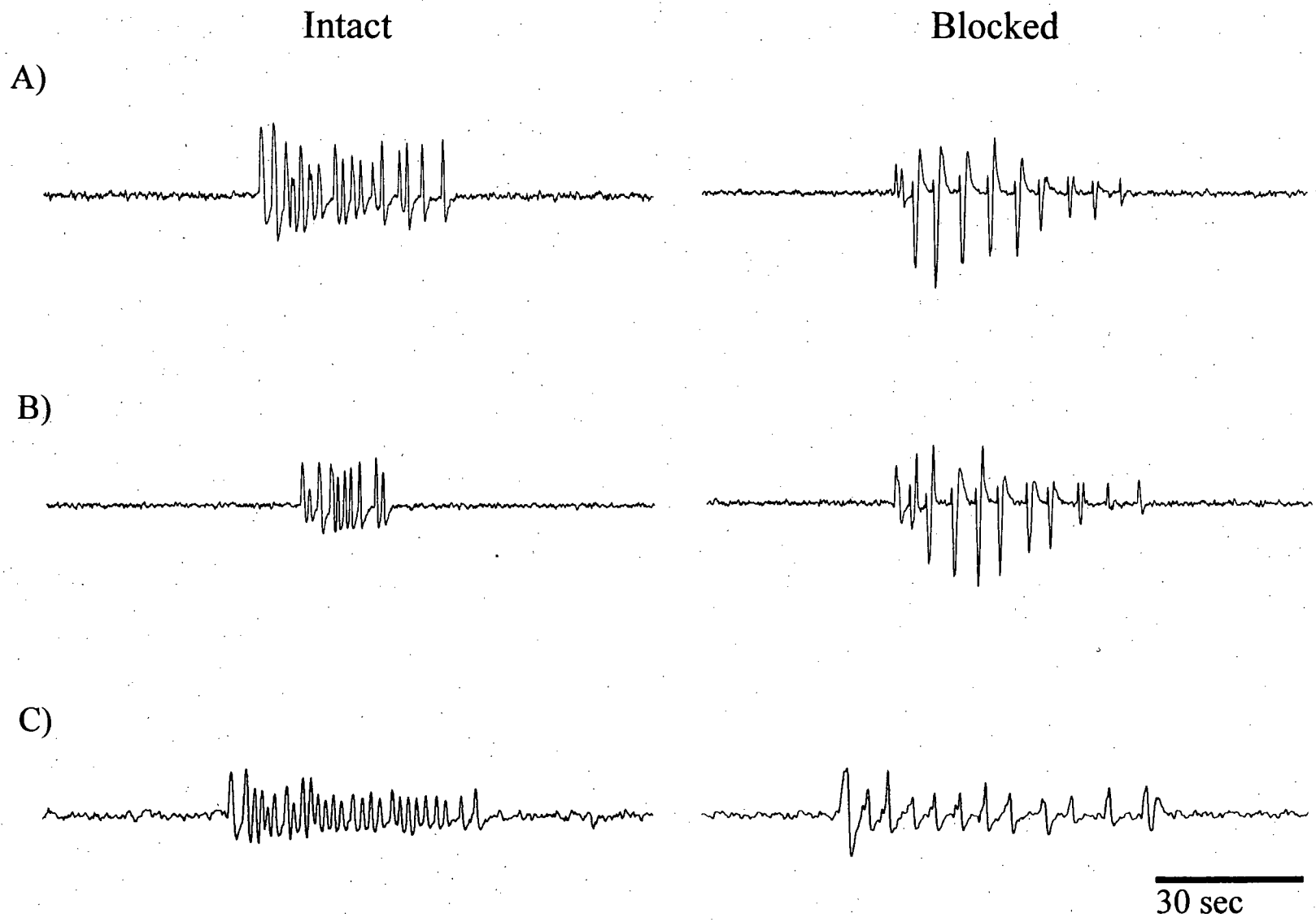

Intact
Blocked
A)
B)
C)
30 sec

Fig. 4.11

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T), ventilation (V_E), breaths per episode, episodes per hour and the instantaneous frequency of breathing within episodes in hibernating animals during exposure to air (open bars), 7% O_2 (hatched bars, rising right) and 5% CO_2 (hatched bars, rising left), before (control) and after (VX) vagal blockade. The "*"denotes a significant difference from control values during exposure to air, while the "#" denotes a significant difference from control values during exposure to the same gas ($P<0.05$).

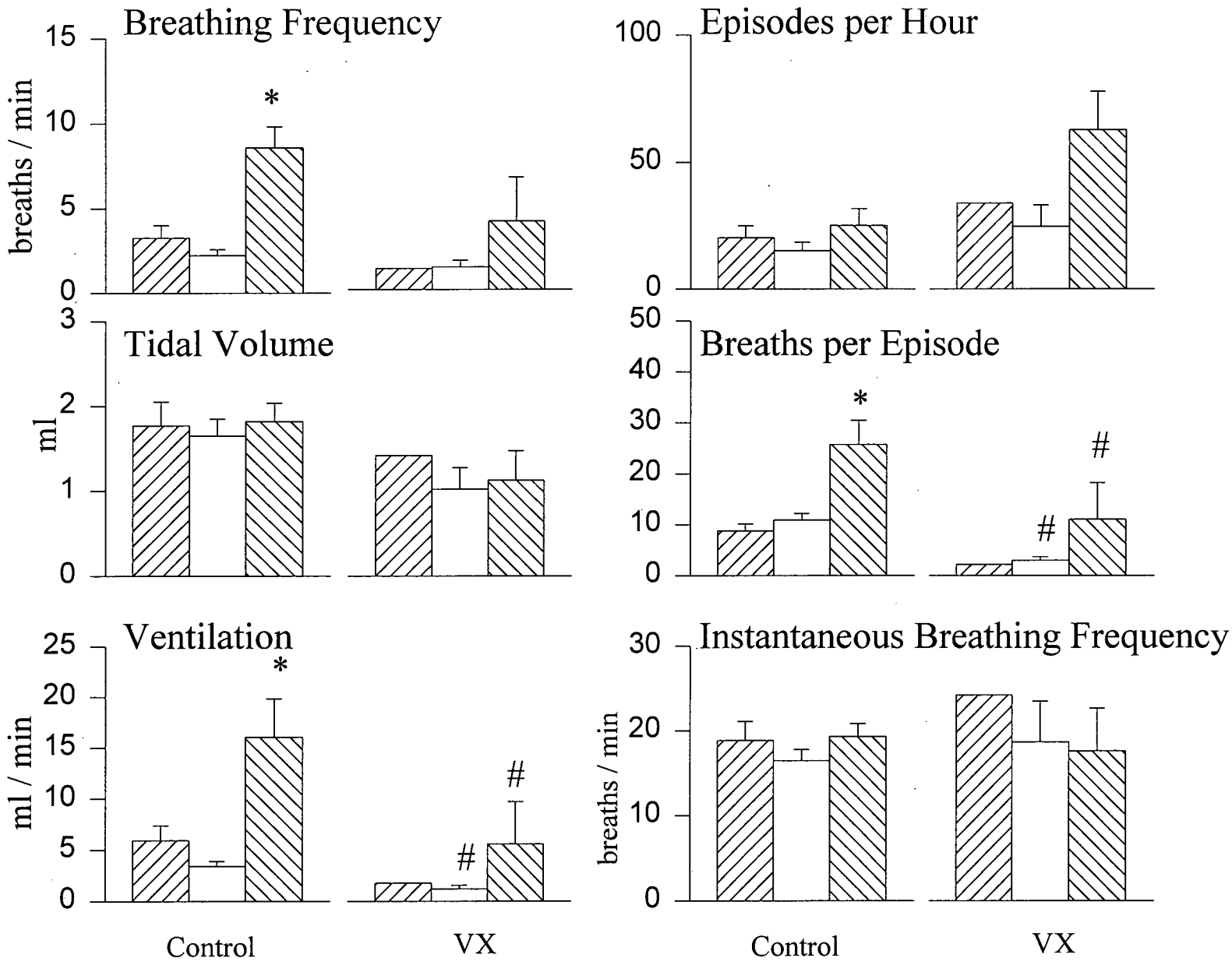
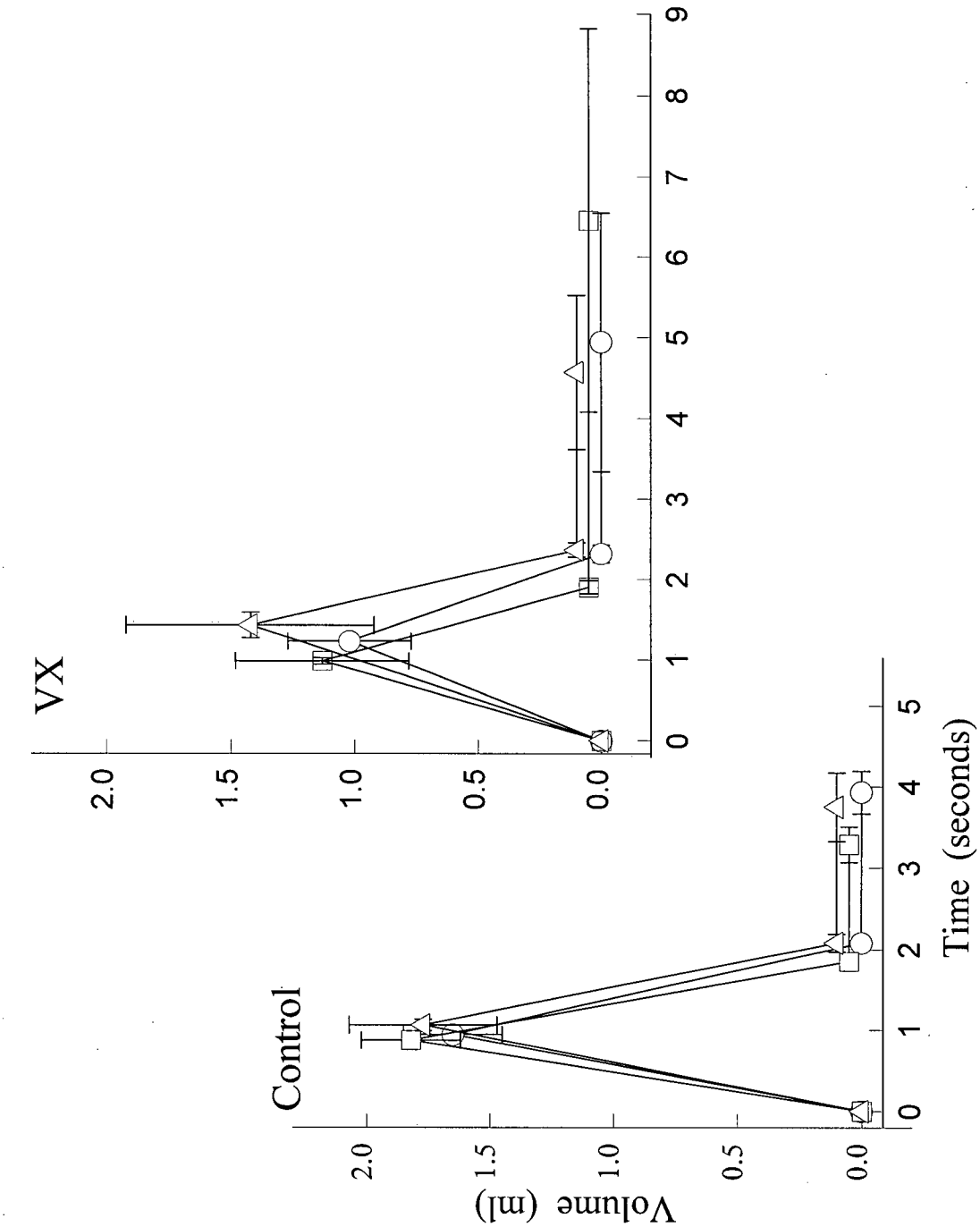

Breathing Frequency
breaths / min
15
10
5
0
*
Episodes per Hour
100
50
0
Tidal Volume
ml
3
2
1
0
Breaths per Episode
50
40
30
20
10
0
*
#
#
Ventilation
ml / min
25
20
15
10
5
0
*
#
#
Instantaneous Breathing Frequency
breaths / min
30
20
10
0
Control
VX
Control
VX

Fig. 4.12

Representation of mean (± standard error) inspiratory volumes and durations of inspiration (TI) and the components of expiration (TEA and TEP) in hibernating animals during exposure to air (○), 7% O_2 (△) and 5% CO_2 (□), before (Intact) and after vagal blockade (VX). Statistical differences are noted in the text.


VX
Control
Volume (ml)
Time (seconds)
0.0
0.5
1.0
1.5
2.0
0
1
2
3
4
5
6
7
8
9

4.4. Discussion

4.4.1. Euthermic animals

4.4.1.1. Ventilatory responses

Hypoxia produced significant increases in $\dot{V}_E$ in both QW and SWS. During QW, this was mediated by apparent yet non-significant increases in both f_R and V_T, while in SWS, it was mediated exclusively by a significant increase in f_R. Hypercapnic exposure produced significant increases in $\dot{V}_E$ in both QW and SWS due to increases in V_T. In QW, f_R was decreased in hypercapnia but remained constant in SWS. These data agree with observations in this (Webb and Milsom, 1994; Hunter et al. 1997) and other species (Hedmark and Kronenberg, 1982; Walker et al. 1985; Cragg and Drysdale, 1983).

Figure 4.3 illustrates the changes in pattern of individual breaths associated with exposure to each gas treatment, during wakefulness and sleep. The significant increase in f_R in hypoxia in SWS was due to reductions in both T_I and T_{EP}. Of note, however, is that while V_T increased significantly in hypercapnia in both QW and SWS, increases in V_T resulted from an increase in mean inspiratory flow rate during SWS with no significant increase in T_I, whereas, during QW, both T_I and mean inspiratory flow rate were significantly increased. This difference most likely reflects the differences in T_I and V_T between QW and SWS observed during exposure to air.

Hypercapnic exposure is generally associated with an increase in V_T produced by an increase in mean inspiratory flow rate and decrease or no change in T_I (Bradley et al. 1974; Cunningham et al. 1986 q.v.). The increases of T_I and mean inspiratory flow rate in QW during hypercapnia, combined with the increase in T_{EP} which decreased f_R, is curious. Prolongation of T_E and reduction of f_R is also an uncommon response to CO_2 (Cunningham et

al. 1986 q.v.). Clark and Von Euler (1972) determined that, when ventilation was stimulated with hypercapnia, the durations of inspiration (TI) and expiration (TEA + TEP) were correlated with each other and inversely related to VT. Thus, it would be expected that VT increased, both TI and TEP should fall so that both fR and VT would rise and augment VE. Observations, similar to those noted here for ground squirrels, have been made in woodchucks and hamsters, however, suggesting that this may be a characteristic of burrowing mammals (Boggs et al. 1992; Tenny and Boggs, 1986). Burrowing animals commonly inhabit hypercapnic environments where elevations of fR do not enhance CO_2 excretion, while increases in VT produce a greater degree of alveolar ventilation. Thus, this seemingly aberrant ventilatory response is appropriate for this species and may represent a significant adaptation (Tenny and Boggs, 1986).

The results presented in this and a previous study (chapter 3) indicate that the states of slow-wave sleep and wakefulness have a significant influence on breathing pattern *per se*. The patterns of ventilation observed during hypoxic exposure, however, were not significantly different between QW and SWS suggesting that this ventilatory chemoresponse overrides the influences of sleep-state. During exposure to hypercapnia, both TI and TEP still increased during the transition between QW to SWS, although, the magnitude of these changes were reduced from those observed during exposure to air. Thus, the hypercapnic ventilatory response appears to dampen but does not completely override the influence of state.

4.4.2. Anesthetized animals

4.4.2.1. Intact

Anesthetized squirrels exhibited ventilatory responses similar to those of unanesthetized

animals. In both State I and III, animals exposed to hypoxia exhibited an increase in V_E, primarily mediated by an increase in f_R. Hypercapnic exposure resulted in a large increase in ventilation mediated by increases in both f_R and V_T in State I, but mediated solely by increases in V_T in State III. These results are similar to those of Hunter et al. (1997).

During each state, the increase in f_R during exposure to hypoxia was produced by a reduction in T_{EP}. In addition, there was a tendency for T_I and V_T to decrease which became significant in State III. These changes are similar to those which occurred in unanesthetized animals in QW and SWS during hypoxia, and agree with studies of others (Kelsen et al. 1977). The increase in V_T during hypercapnia stemmed from an increase in inspiratory flow while T_I was not significantly altered. This was similar to the influence of hypercapnia in SWS.

Overall, the hypoxic response of anesthetized animals was less robust than that of unanesthetized animals, while the hypercapnic response was as great as, or greater than that seen in unanesthetized animals. Despite these discrepancies, hypoxia and hypercapnia both had powerful influences on breathing pattern. Transitions between States I and III had a significant influence on breathing pattern which could either counter or compliment the effects of hypoxia or hypercapnia, just as was observed during transitions between QW and SWS in unanesthetized animals. The increases in T_I, V_T and T_{EP} associated with transitions from States I to III were lost, however during hypoxic exposure, while differences in T_I and T_{EP} persisted during hypercapnia. Thus, as was observed in unanesthetized animals, the effects of these gases appear to be powerful and to override, at least in part, the effects of transitions between states.

4.4.2.2. Post - vagotomy

Following vagotomy, V_T was greatly increased and f_R depressed in both States I and III in animals breathing air. Although overall ventilation was not altered, it was much more variable. Both hypoxia and hypercapnia resulted in non-significant increases in f_R and V_T during States I and III following vagotomy. None-the-less these changes resulted in increases in ventilation during hypoxia and hypercapnia that were significant at $P<0.05$ in State I, but only at $P<0.10$ during State III. The only significant alteration of pattern of individual breaths in response to changes in gas treatment following vagotomy, was a decrease in T_{EP} associated with hypoxia during States I and III. There were no changes in breathing pattern associated with transitions between states on any gas. Unanesthetized dogs exhibit similar increases in V_T and decreases in f_R following vagotomy, yet they respond equally to hypoxia before and after this treatment (Xi et al. 1993). Their responses to hypercapnia, however, are impaired (Phillipson et al. 1973).

One of the initial hypotheses of the present investigation was that vagal feedback would influence the effect of changes in blood gases on both breathing pattern and the magnitude of the ventilatory responses in anesthetized animals. The results support this hypothesis in part and indicate that feedback from pulmonary receptors does influence the expression of ventilatory chemoresponses in anesthetized squirrels. This feedback also influences the changes in ventilation associated with changes in activation state. The hypoxic ventilatory response, which is primarily an increase in f_R, is not greatly influenced by vagotomy but the hypercapnic ventilatory response, which is primarily an increase in V_T, is greatly reduced. V_T following vagotomy was slightly less than the total vital capacity reported in this species (Milsom and Reid, 1995). Thus, V_T may have already been maximum, precluding further

changes due to hypoxia or hypercapnia. Similar impairment of the hypercapnic response is not seen following vagotomy in either anesthetized or unanesthetized rats (Martin-Body and Sinclair, 1987). These rats did not exhibit as great an increase in VT following vagotomy itself and, thus, VT may not be under the same constraints in rats as it is in squirrels. In general, however, these results are similar to those obtained for other species (Chapman et al. 1982; Chonan et al.1990; Guz et al. 1966; Martin-Body and Sinclair, 1987; Richardson and Widdicombe, 1969; Xi et al. 1993) and are consistent with our predictions.

4.4.3. Hibernating animals

4.4.3.1. Intact

Hibernating squirrels exhibited an extremely robust ventilatory response to hypercapnia (370% increase in VE), when compared to that of euthermic animals (63% increase), consistent with previous investigations (McArthur and Milsom, 1991b). Also consistent with previous studies, animals in the present investigation did not exhibit a significant hypoxic ventilatory response. Previous studies have indicated that the hypoxic ventilatory response of this species is drastically reduced during hibernation and does not occur until inspired O_2 is decreased to 5% or less (McArthur and Milsom, 1991b). Lower concentrations of O_2 were administered during the initial stages of this investigation but steady state measurements were not possible as levels of hypoxia below 6% O_2 often stimulated the animals to arouse from hibernation.

4.4.3.2. Post - vagal blockade

The increase in ventilation exhibited by ground squirrels during hypercapnic exposure, was due primarily to increases in the number of breaths in each episode. Small, non-significant

increases in the frequency of breathing episodes and the number of breaths occurring in each episode were all that remained following vagal blockade. Since we have shown previously that the primary effect of vagotomy in hibernating squirrels is a reduction in the size of breathing episodes, and since the primary response of hibernating squirrels to hypercapnia is an increase in the size of breathing episodes, the absence of a hypercapnic ventilatory response, post vagal block, is not surprising.

The similarity between the hypercapnic ventilatory responses of intact hibernating ground squirrels (Harris and Milsom, 1994) and vagotomized rabbits (Richardson and Widdicomb, 1969), and the observation that some vagally mediated reflexes may be absent during hibernation (Lyman, 1982) lead to a null hypothesis that feedback from pulmonary receptors does not modulate ventilatory chemoresponses during hibernation. Given the results of a previous investigation, which demonstrated that vagotomy significantly altered breathing pattern (Chapter 3), however, it was predicted that vagal feedback would influence the manner in which changes in blood gases alter the episodic breathing pattern in hibernating squirrels. The results of the present investigation support this hypothesis and indicate that vagal afferent information does significantly modulate the hypercapnic ventilatory response of these animals during hibernation. As a consequence the cause of the ventilatory plateau present in the hypercapnic response prior to vagotomy remains enigmatic.

4.5. Conclusion

It was predicted that vagal feedback would influence the effect of changes in blood gases on both breathing pattern and the magnitude of the ventilatory responses in euthermic and hibernating squirrels

The data from euthermic animals indicate that hypoxic and hypercapnic ventilatory responses are essentially similar between anesthetized and unanesthetized animals. In anesthetized animals, these ventilatory responses are disrupted but not eliminated by vagotomy. Thus, the interaction between the chemoreceptor and pulmonary afferent influences on breathing is complimentary rather than conditional in euthermic animals.

During hibernation, hypercapnia stimulates the occurrence of breaths in individual episodes. Removal of vagal feedback from pulmonary receptors depresses the occurrence of breaths in each breathing episode, and this effect is not overcome by subsequent hypercapnic exposure. Thus, these data indicate that the importance of pulmonary receptor feedback during hibernation is equal to or greater than it is during euthermia.

Chapter 5

The Influence of NMDA Receptor-Mediated Processes on Breathing Pattern in Ground Squirrels

Abstract

The effects of blockade of the function of N-methyl-D-aspartate (NMDA) type glutamate receptors by the non-competitive antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801) on breathing pattern and cortical arousal were assessed in the golden-mantled ground squirrel, *Spermophilis lateralis,* during sleep and wakefulness, anesthesia, and natural hibernation.

Before treatment, unanesthetized animals cycled between sleep and wakefulness, while anesthetized animals cycled between states with cortical activity patterns similar to sleep and wakefulness. Breathing was depressed during anesthesia and was lower in sleep and sleep-like states than in wakefulness and wake-like states. Unanesthetized animals did not sleep following treatment with MK-801, and their breathing pattern was similar to that normally observed during wakefulness. Treatment with MK-801 prevented the expression of the sleep-like state normally observed in anesthetized animals, resulting in a constant wake-like state. This treatment stimulated breathing frequency beyond that observed during the wake-like state to levels equal to those observed in unanesthetized animals during wakefulness.

Hibernating animals breathed in distinct multi-breath episodes separated by prolonged apneas. Treatment with MK-801 increased the number of breaths in each breathing episode and the frequency of breathing during these episodes, but did not alter overall ventilation. These data suggest that there are NMDA-mediated processes which facilitate the expression of sleep in unanesthetized animals and sleep-like states in anesthetized animals, and which depress breathing frequency during sleep, anesthesia and hibernation.

5.1. Introduction

It is generally accepted that the basic rhythm of breathing in mammals is generated by a "central rhythm generator" (CRG) localized within the brainstem that can function independent of modulatory inputs (for review see Bianchi et al. 1995). This basic rhythm, however, is shaped by various inputs to create complex breathing patterns (Feldman et al. 1990; Bonham, 1995 q.v.). The CRG and its modulatory inputs act as a functional "central pattern generator" (CPG) and determine the duty cycle, timing of inspiration and expiration, amplitude of the breath, and effectively match ventilation to ventilatory demands.

In chapter 3 it was demonstrated that while unanesthetized animals cycled between sleep and wakefulness, anesthetized animals cycled between states with cortical activity patterns similar to sleep and wakefulness. It was also determined that these alterations in state had significant influences on breathing, specifically, that tidal volume increased, breathing frequency decreased and the timing components of individual breaths changed as animals cycled from awake and "wake-like" to sleep and "sleep-like" states, respectively. As such, these states constitute modulatory inputs which act as part of the CPG.

It has long been recognized that supramedullary inputs influence breathing pattern. In particular a region of the pons, consisting of the medial and lateral Parabrachial and Kölliker-Fuse nuclei (the Parabrachial complex, PBC), is well known to modulate breathing pattern and to act as part of the CPG (see Bianchi et al. 1995 for review).

Autoradiographic and immunocytochemical surveys (Monaghan and Cotman, 1985; Petralia et al. 1994) have shown that the PBC contains a high proportion of the N-methyl-D-aspartate (NMDA)-type receptors for the excitatory amino acid glutamate, and that this receptor type is otherwise relatively scarce in midbrain and brainstem regions. Numerous studies have

demonstrated that those functions of the PBC associated with ventilation are mediated through NMDA-type receptors, whereas the rhythmogenic properties of the CRG and reflex ventilatory responses such as the Breuer-Hering reflex do not involve NMDA receptor-mediated processes (Karius et al. 1991; Funk et al. 1993; Bonham, 1995 q.v.). Disruption of the function of NMDA-type receptors with specific antagonists has, thus, been used to investigate NMDA receptor-mediated influences of the PBC (Connelly et al. 1992; Ling et al. 1993; Fung et al. 1994; Cassus-Soulanis et al. 1995; Bonham, 1995 q.v.).

With the advent of *in vitro* preparations for the study of respiratory neural networks, rodents have become an important model for the study of respiration. It has been suggested that the rodent respiratory control network is identical to that of the cat (Wang et al. 1994; Fung et al. 1994). Rodents, however, have not been demonstrated to react as uniformly to antagonism of NMDA receptor function as have cats (Montreau et al. 1990; Connelly et al. 1992; Cassus-Soulanis et al. 1995; St.John, 1996). These conflicting reports bring into question the relative importance of NMDA receptors and of the PBC itself in the control of breathing in rodents, and suggest that there could be significant species and strain-specific differences between rodent types.

The same autoradiographic and immunocytochemical surveys which identified NMDA receptors within the PBC also identified these receptors within the pontine reticular formation (Monaghan and Cotman, 1985; Petralia et al. 1994). This region is involved in the generation of states of central activation and contributes to the influences that such states have on ventilation (see Bianchi et al. 1995; Phillipson and Bowes, 1986 for review). As such, NMDA receptor antagonism may have a secondary influence on ventilation stemming from a primary influence on central activation state. This potentially confounding factor is rarely taken into

account as few studies measure EEG or consider central activation state.

To allow broad comparisons, the present investigation was designed to assess the influences of NMDA-type glutamatergic blockade on central activation state and the generation of breathing pattern in spontaneously breathing golden-mantled ground squirrels during unanesthetized sleep and wakefulness, anesthesia and natural hibernation.

5.2. Methods

5.2.1. Instrumentation

Experiments were performed on a total of twenty five adult golden-mantled ground squirrels *(Spermophilus lateralis)* of mixed sex. Animals used in the chronic studies were surgically instrumented *a priori*. Animals were fitted with four electroencephalographic (EEG), two electrocardiographic (ECG), two electromyographic (EMG) and two respiratory impedance electrodes, and a third cerebral ventricle cannula (Boswell et al. 1993) as outlined in the general methods section (chapter 2).

5.2.2 Protocol

5.2.2.1. Euthermic animals (chronic study)

Six squirrels selected for this portion of the study underwent surgery during the summer and were held for at least three weeks following surgery before experimentation.

Unanesthetized animals were transferred to a whole body plethysmograph. The "dummy" cannula was replaced with a 23-gauge "injection" cannula (Plastics One , Roanoke VA.) which was connected to a 10 µl Hamilton syringe via a length of polyethylene tubing (PE 20). The syringe and tubing were filled with a solution of the noncompetitive NMDA-type glutamatergic blocker, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801, Research Biochemicals) in saline (5 mg/ml, 15 mM; pH 7.3 ±0.1) except for the final 2 µl at the tip which was filled with saline, separated from the MK-801 solution by a small (<0.2 µl) air bubble.

Animals were monitored for at least 2 hours before any manipulation, for 30 minutes following a 2 µl injection of saline (sham) into the third ventricle, and for 30 minutes following

a 2 µl injection of the MK-801 solution. Additional injections of MK-801 solution were repeated every 30 to 45 minutes.

5.2.2.2. Hibernating animals

Twelve animals selected for this portion of the study underwent surgery during the winter. Following recovery, these animals reentered hibernation. During deep hibernation, animals were transferred to a plexiglass box (12 x 20 x 10 cm) within a laboratory controlled-environment chamber. The box was supplied with air at a flow rate of 500 ml per minute. Ambient temperature was held at 5 ± 1 °C. Animals were fitted with a mask connected to a resistance pneumotachograph to measure ventilation. The "dummy" cannula was replaced with a 23-gauge "injection" cannula (Plastics One , Roanoke VA.) which was connected to a 10 µl Hamilton syringe as described above.

Animals were monitored; i) for at least two hours to provide initial control values, ii) for at least one hour following a 2 µl injection of saline (sham treatment) into the third ventricle, iii) for at least one hour following a 2 µl injection of the MK-801 solution, and iv) for one hour periods following additional 2 µl injections of the MK-801 solution.

5.2.2.3.. *Anesthetized animals (acute study)*

Seven ground squirrels were used in this series of experiments. Animals were anesthetized with urethane, tracheotomized, fitted with EEG, ECG, EMG and respiratory impedance electrodes and had catheters implanted in a femoral artery and vein, as described in Chapter 2. Animals were monitored; i) for at least two hours to provide initial control values, ii) for at least one hour following a 0.3 ml intravenous infusion of saline (sham) into the

venous catheter, iii) for one hour following a 0.2 to 0.3 ml infusion of a 0.15 mg/ml (0.45 mM) solution of MK-801 in saline (pH 7.3 ±0.1) resulting in a dose of 0.15 - 0.30 mg MK-801 per kg body mass and, iv) for additional one hour periods following two additional 0.2 to 0.3 ml infusions of the same MK-801 solution.

5.2.2.4. *Additional anesthetized animals*

The anesthetized animals just described received MK-801 through intravenous injection, while those used in the unanesthetized studies received MK-801 treatment via direct injection into the cerebral-spinal fluid. This drug easily crosses the blood-brain barrier, and is commonly administered by intravenous or intra peritoneal injection (Cassus-Soulanis et al. 1995). To assess the potential difference which route of drug infusion may have had on the results three of the animals instrumented and used in the unanesthetized portion of this series were subsequently tested during anesthesia. These animals were anesthetized with a 5ml/kg intra peritoneal injection of a 20% solution of urethane, supplemented with vaporous halothane. The trachea was cannulated, halothane was discontinued and the animals were transferred to the homeothermic table within the electrically shielded chamber. The tracheal catheter was connected to a ventilatory pneumotachograph and air line and the animals left to stabilize for approximately two hours. Arterial and venous catheters were not implanted in these animals, and EEG, EMG, and EKG were monitored using the previously implanted electrodes.

Animals were monitored; i) for at least two hours to provide initial control values, ii) for at least one hour following a 2 μl injection of saline (sham treatment) into the third ventricle, iii) for at least one hour following a 2 μl injection of the MK-801 solution, and iv) for one hour periods following additional 2 μl injections of the MK-801 solution.

5.3. Results

5.3.1 Euthermic animals

5.3.1.1. Control conditions

As reported in a chapter 3, animals regularly cycled between periods of quiet wakefulness (QW) and slow-wave sleep (SWS), interspersed occasionally with periods of a state defined as "active" wakefulness (AW) (Fig. 5.1). Although present, paradoxical or rapid-eye-movement sleep (REM) occurred only in short (<45 second) and irregular bouts. Ventilation was analyzed only during well established periods of QW and SWS. Breathing during AW, REM and the various transition phases was ignored.

Transitions between the states of QW and SWS had significant influences on breathing. Animals exhibited an increase in tidal volume (V_T) (from 2.34 ± 0.28 to 5.17 ± 1.12 ml) and decrease in respiratory frequency (f_R) (from 129.5 ± 9.21 to 51.3 ± 4.7 br/min) as they progressed from QW to SWS (Fig. 5.2). There was, however, no significant change in ventilation (V_E) (319.4 ± 55.1 *vs.* 259.5 ± 44.9 ml/min) between these states. Changes in breathing pattern resulted from increases in the durations of inspiration (T_I), expiration (T_E) and, notably the end-expiratory pauses (T_{EP}) following the period of expiratory flow (T_{EA}) between states (Fig. 5.2).

5.3.1.2. Post - MK-801 treatment

Injections of saline into the cerebral-spinal fluid had no immediate influence on EEG or breathing. Within 10 minutes of MK-801 treatment, however, all animals entered a period of continuous QW. Following MK-801 treatment, SWS never occurred, and QW was interrupted, only occasionally, by brief periods of AW. The incidents of AW appeared to be

Fig. 5.1

Representative recordings of EEG and breathing of an unanesthetized squirrel during A) quiet wakefulness, B) slow-wave sleep (SWS) and C) following MK-801 treatment. Breathing records are from the output of the plethysmograph, with inspiration as an upward deflection and tidal volume proportional to the area under the deflection.

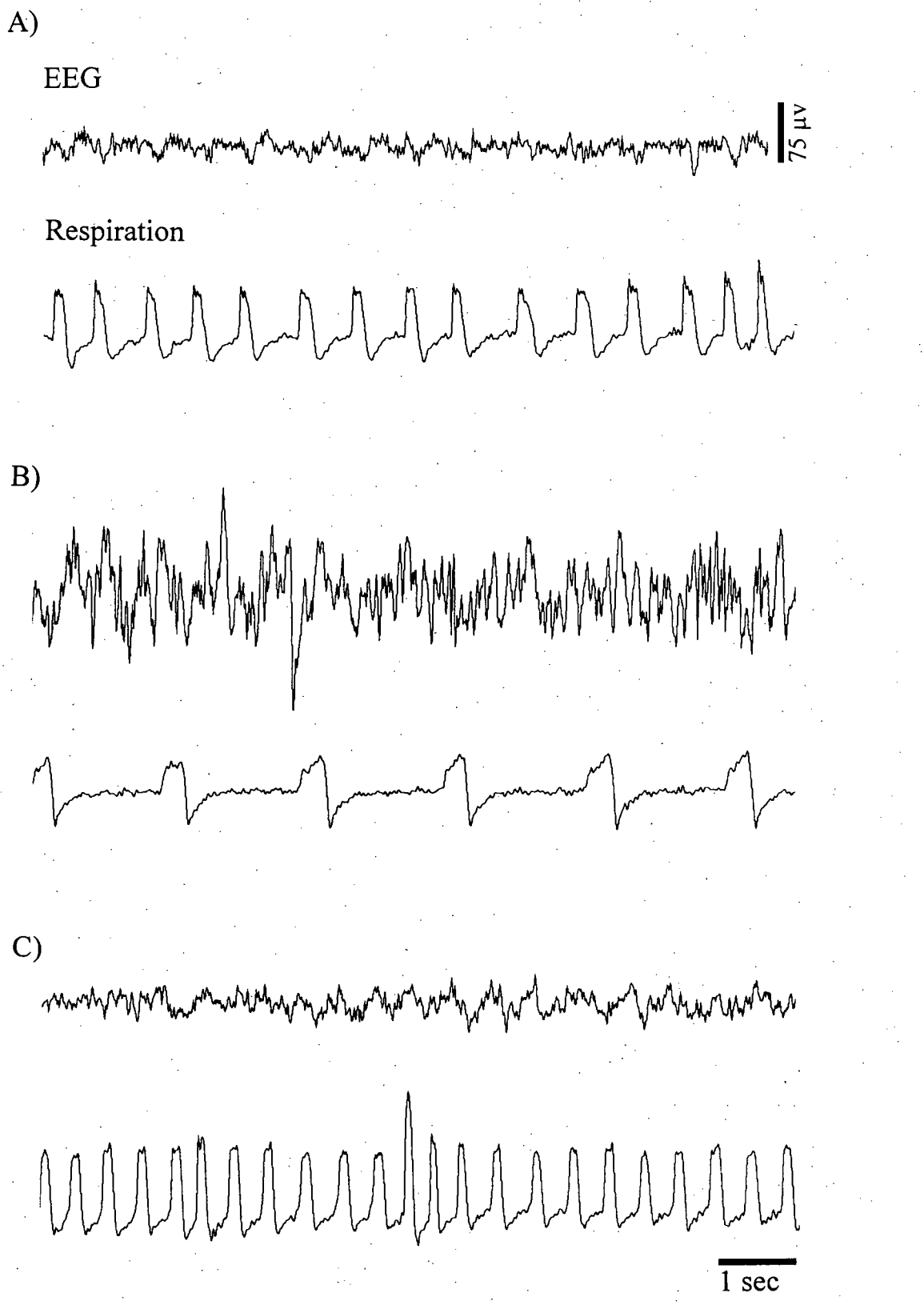
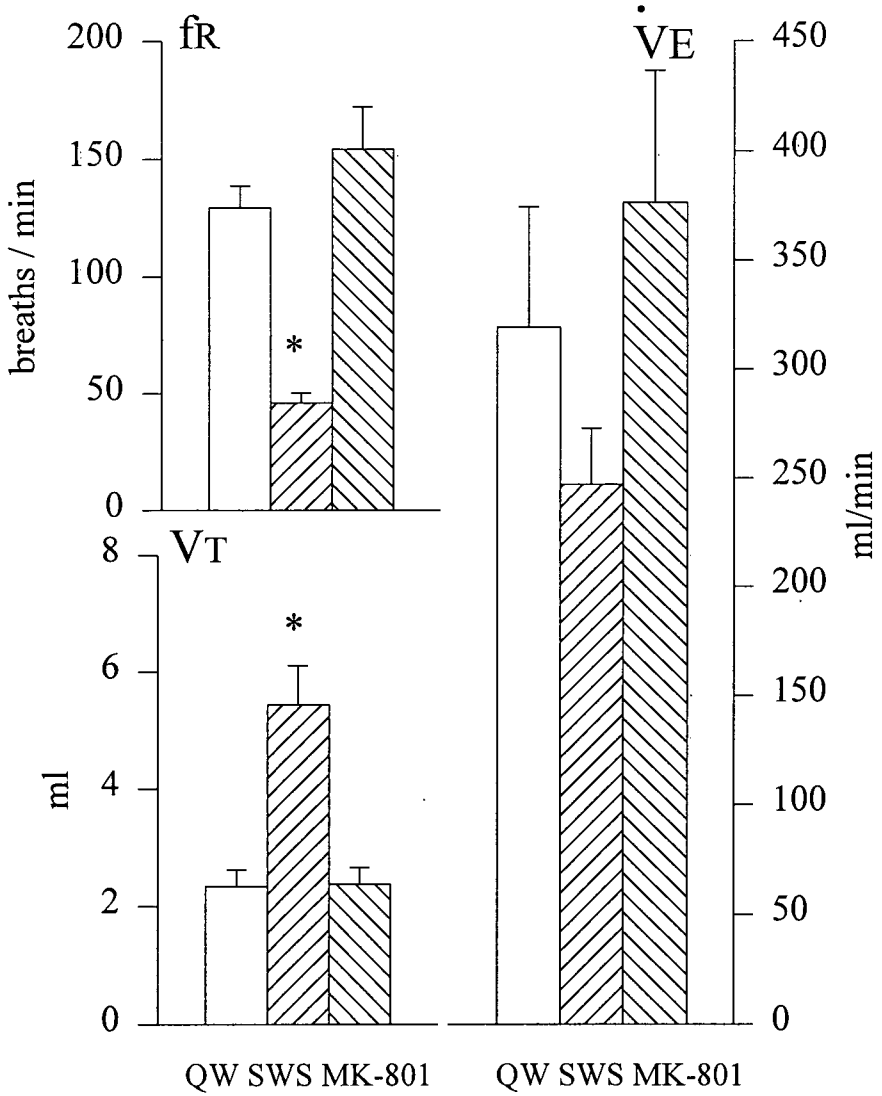
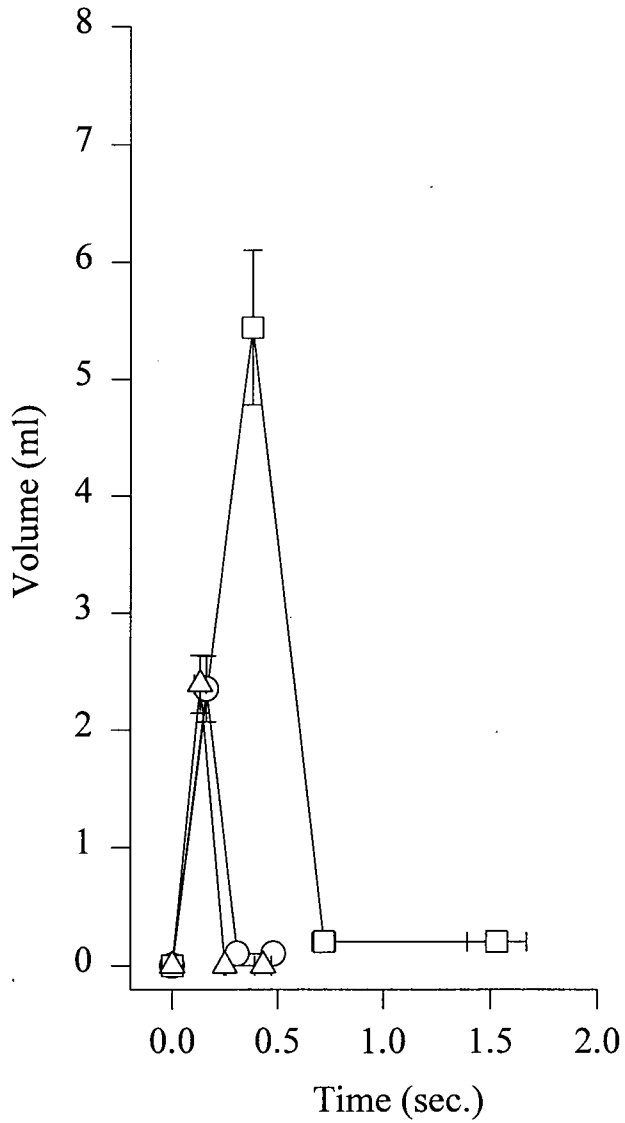

A)
EEG
75 µv
Respiration
B)
C)
1 sec

Fig. 5.2

The left panel illustrates mean (± standard error) values for; breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E), during quiet wake (open bars), slow-wave sleep (hatched bars rising right), and following MK-801 treatment (hatched bars rising left). The "*" denotes a significant difference from quiet wake values in the left panels ($P<0.05$), while statistical differences in the data to the right are noted in the text.

The right panel illustrates mean (± standard error) values for; inspiratory volumes and durations of inspiration and expiration of unanesthetized animals during quiet wake (○), slow-wave sleep (□), and following MK-801 treatment (△). Expiration is composed of a period of expiratory flow (T_{EA}) and an end-expiratory pause (T_{EP}). Statistical differences in the data are noted in the text. Also note that in different plots the end-expiratory volumes have been offset from zero for clarity.


fR
breaths / min
200
150
100
50
0
*
$\dot{V}E$
ml/min
450
400
350
300
250
200
150
100
50
0
VT
ml
8
6
4
2
0
*
QW SWS MK-801
QW SWS MK-801


Volume (ml)
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Time (sec.)

less frequent than before MK-801 treatment, but this was not assessed.

Breathing following MK-801 treatment, was not significantly different from that observed during QW (Fig. 5.1). Animals exhibited a mean (± standard error) f_R of 154.3 ± 18.1 breaths per minute, a V_T of 2.38 ± 0.28 ml, resulting in a $\dot{V}_E$ of 376.6 ± 60.7 ml per minute (Fig. 5.2). There were also no significant differences in the timing of individual breaths. Additional doses of MK-801 did not alter either the central activation state or breathing further.

5.3.2 Anesthetized animals

5.3.2.1 Control conditions

As reported in chapter 3, animals under urethane anesthesia exhibited an "awake-like" (State I) and a "slow-wave-sleep-like" (State III) state of central activation, defined by analysis of the EEG waveform (Fig. 5.3). Like animals in natural sleep and wakefulness, anesthetized animals exhibited alterations in breathing pattern associated with states of central activation. Animals exhibited a decrease in f_R (from 56.2 ± 4.0 to 31.9 ± 3.6 br/min) as they progressed from State-I to State-III (Fig. 5.4). There were, however, no significant differences in V_T (1.35 ± 0.2 vs. 2.03 ±0.3 ml) or $\dot{V}_E$ (75.3 ± 12.1 vs. 66.0 ±13.2 ml/min) between these states. Decreases in f_R resulted from increases in T_I and T_{EP} between states, whereas T_{EA} was unchanged (Fig. 5.5).

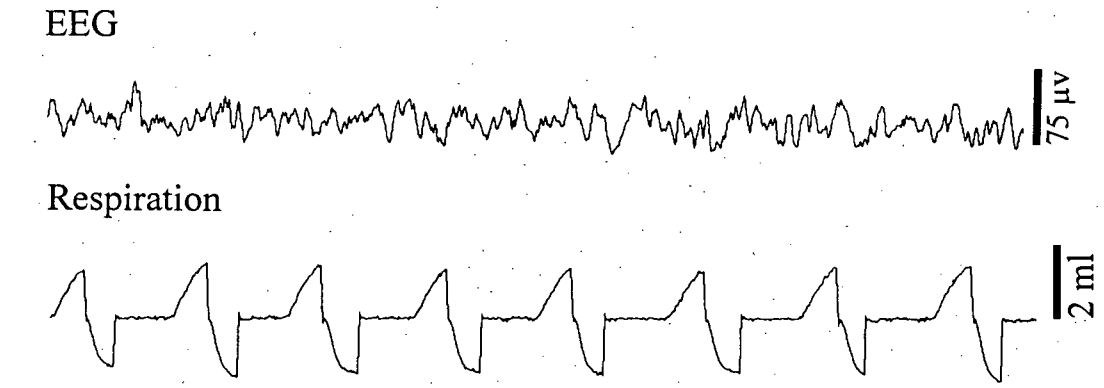
5.3.2.2. Post - MK-801 treatment (intravenous)

Intravenous injections of saline did not alter either central activation state or breathing. Within 10 minutes of MK-801 treatment, however, all animals exhibited a consistent State-I type EEG which did not change for the remainder of the experiment.

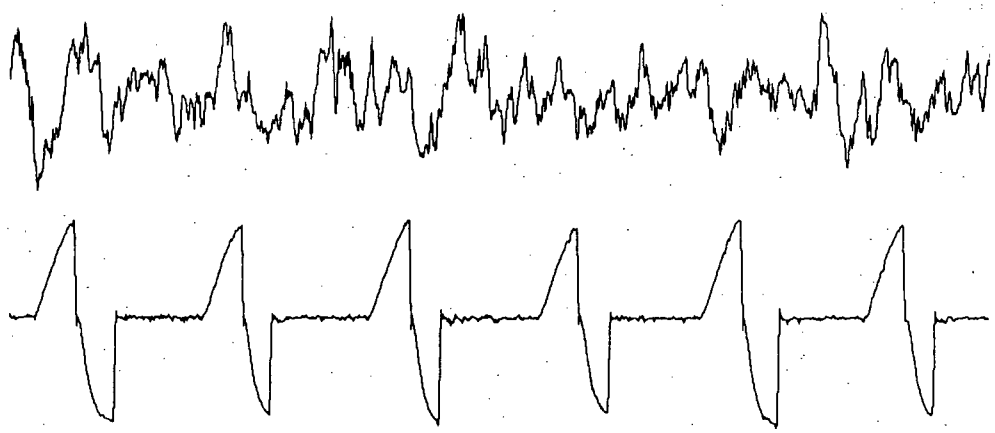
Fig. 5.3

Representative recordings of EEG and breathing of an anesthetized squirrel during A) State I, B) State III and C) following MK-801 treatment. Breathing records are the integrated output of the pneumotachograph with inspiration as an upward deflection and peak height proportional to tidal volume.

A)



B)



C)

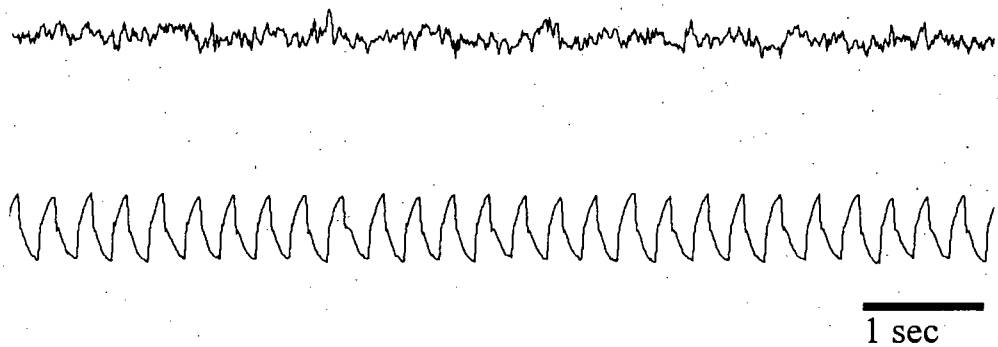


Fig. 5.4

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) in; (left panels) anesthetized animals during State I (open bars), State III (hatched bars rising right), and following intravenous MK-801 administration (hatched bars rising left) and in a second group of anesthetized animals (right panels) during State I, State III and following administration of MK-801 into cerebral-spinal fluid. The "*" in the left panels denotes a significant difference from State-I values ($P<0.05$), while the low number of subjects ($N=3$) precludes valid statistical assessment of these data in the right panels.

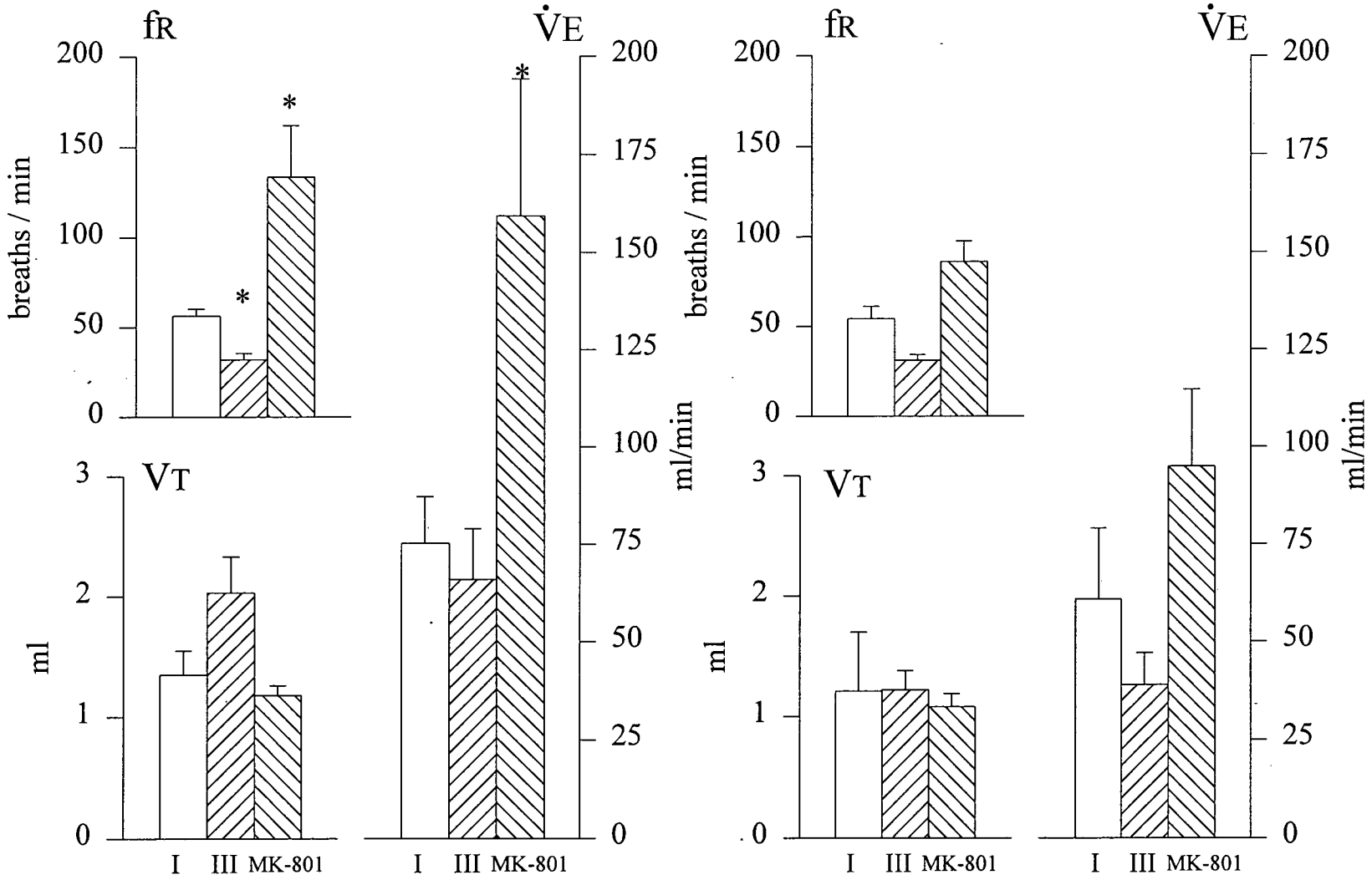
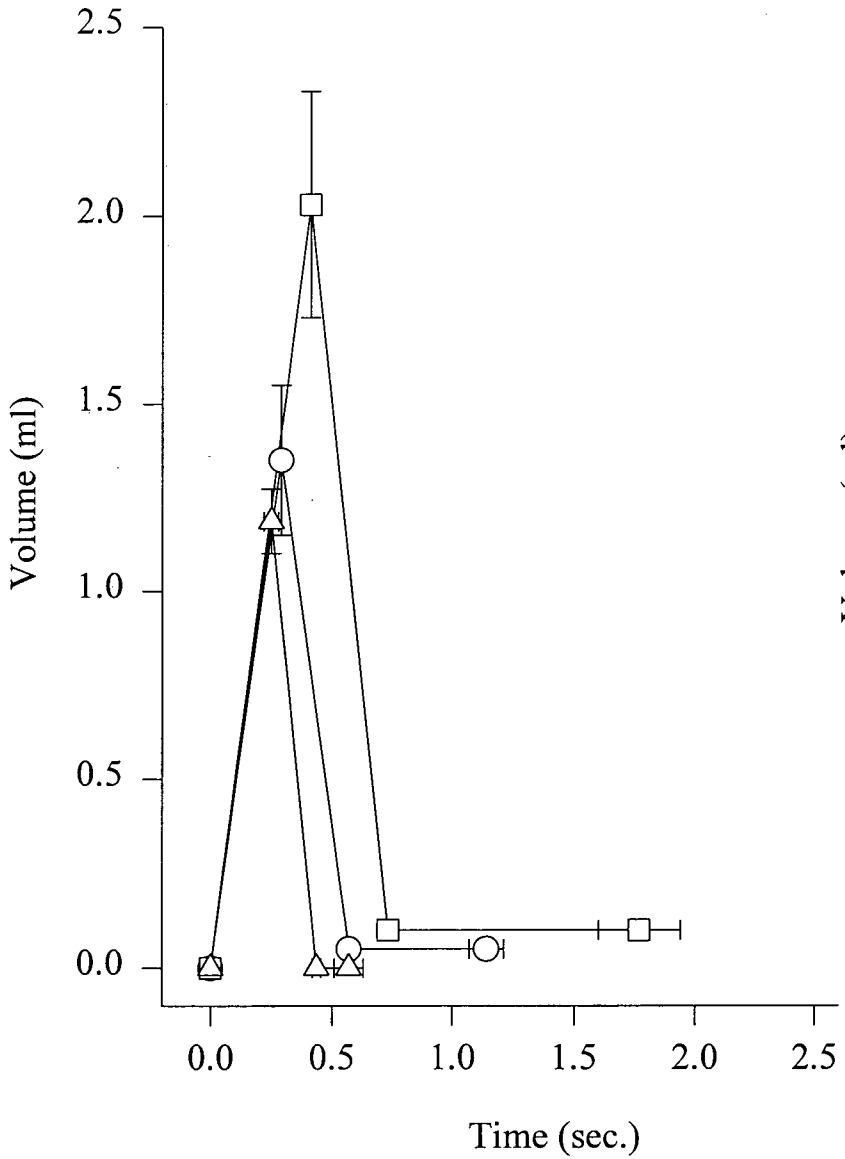
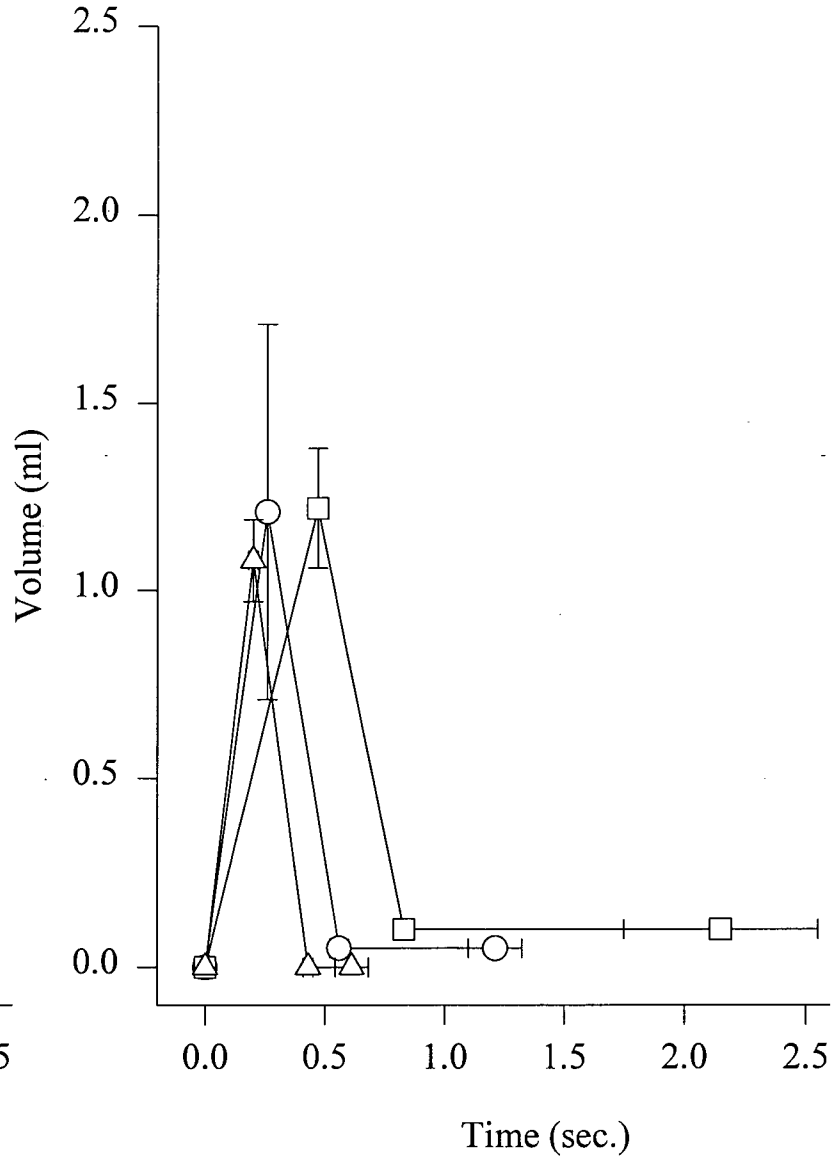

fR
breaths / min
200
150
100
50
0
*
*
VE
ml/min
200
175
150
125
100
75
50
25
0
*
VT
ml
3
2
1
0
I
III
MK-801
fR
breaths / min
200
150
100
50
0
VE
ml/min
200
175
150
125
100
75
50
25
0
VT
ml
3
2
1
0
I
III
MK-801

Fig. 5.5

Representation of mean (± standard error) inspiratory volumes and durations of inspiration and expiration of anesthetized animals (left panels) during State I (○), State III (□), and following intravenous MK-801 administration (△), and in a second group of anesthetized animals (right panels) during State I, State III and following administration of MK-801 into cerebral-spinal fluid. Statistical differences are noted in the text.


2.5
2.0
1.5
1.0
0.5
0.0
Volume (ml)
0.0
0.5
1.0
1.5
2.0
2.5
Time (sec.)


2.5
2.0
1.5
1.0
0.5
0.0
Volume (ml)
0.0
0.5
1.0
1.5
2.0
2.5
Time (sec.)

Breathing was altered by MK-801 treatment. Animals exhibited a dramatic increase in f_R. When compared to State I before MK-801, there was an 138% increase in f_R (from 56.2 ± 4.0 to 133.6 ± 28.5 br/min), while there was a noticeable but non-significant decrease in V_T (from 1.35 ± 0.21 to 1.18 ±0.12 ml). There was, thus, a 116 % increase in overall $\dot{V}_E$ (from 75.3 ± 12.1 to 159.3 ± 35.3 ml/min, Fig. 5.4). The increase in f_R resulted from decreases in T_I and T_{EP}, while T_{EA} and mean inspiratory flow rate were unchanged from pretreatment conditions (Fig. 5.5). Additional doses of MK-801 did not alter either the constant expression of State I EEG or breathing.

5.3.3. *Post - MK-801 treatment (csf)*

Administration of MK-801 directly into the cerebrospinal fluid, rather than into the peripheral circulation also resulted in a transition to, or maintenance of, a State I pattern of EEG. This State I pattern was fully established within 5 minutes of MK-801 treatment. When compared to the obtained values during State I before the treatment, MK-801 treatment produced an increase in f_R (from 54.3 ± 6.9 to 86.1 ± 11.5 br/min) and a decrease in V_T (from 1.21 ± 0.5 to 1.08 ± 0.12 ml), resulting in an increase in $\dot{V}_E$ (60.83 ± 18.2 to 94.9 ± 19.9 ml/min). The increase in f_R resulted from decreases in T_I, T_{EA} and T_{EP}. (Fig. 5.5). The low number of replicates precludes valid statistical assessment of these data. Subjectively, these results appear similar to those observed following intravenous injections of MK-801 in the other anesthetized animals, although f_R and $\dot{V}_E$ were not elevated to the same degree. Again, additional injections of MK-801 solution did not alter EEG pattern or breathing further. The influences of MK-801 treatment appeared to subside after approximately four hours, although objective assessment of this recovery period was not made.

5.3.4 Hibernating animals

5.3.4.1 Control conditions

The EEG of animals hibernating with a body temperature of approximately 7°C showed little discernable activity. The ECG was clearly evident in the EEG. Ventilation in these animals occurred in distinct multi-breath episodes separated by prolonged periods of apnea (Fig. 5.6).

5.3.4.2. Post - MK-801 treatment

Injections of saline into the cerebral-spinal fluid of hibernating animals had no effect. Injection of MK-801 solution did not alter overall ventilation but did result in an alteration of breathing pattern. There was a 40 % increase in the number of breaths occurring in each breathing episode (from 10.9 ± 1.3 to 15.3 ± 2.5 br/ep). This alteration did not result in an increase in ventilation as it was accompanied by a non-significant decrease in the frequency of episodes themselves (from 15.2 ± 3.4 to 9.4 ± 1.4 ep/hr) (Fig. 5.7). The timing of individual breaths was altered only by a significant decrease in TEP following MK-801 (Fig. 5.8). This change resulted in a 25 % increase in the instantaneous frequency of breaths within breathing episodes (from 16.5 ± 1.3 to 20.6 ± 0.8 br/min) (Fig. 5.7).

Additional doses of MK-801 did not alter breathing further in hibernating animals. In some instances, primary or subsequent MK-801 treatment appeared to induce the animals to arouse from hibernation. In all of these instances, the alterations in breathing pattern normally associated with MK-801 treatment preceded induction of arousal by at least two hours.

Fig. 5.6

Representative recordings of breathing from a hibernating squirrel before (top tracing) and after (bottom tracing) MK-801 treatment. Breathing records are the integrated output of the pneumotachograph with inspiration as an upward deflection. Tidal volume is proportional to the peak height.

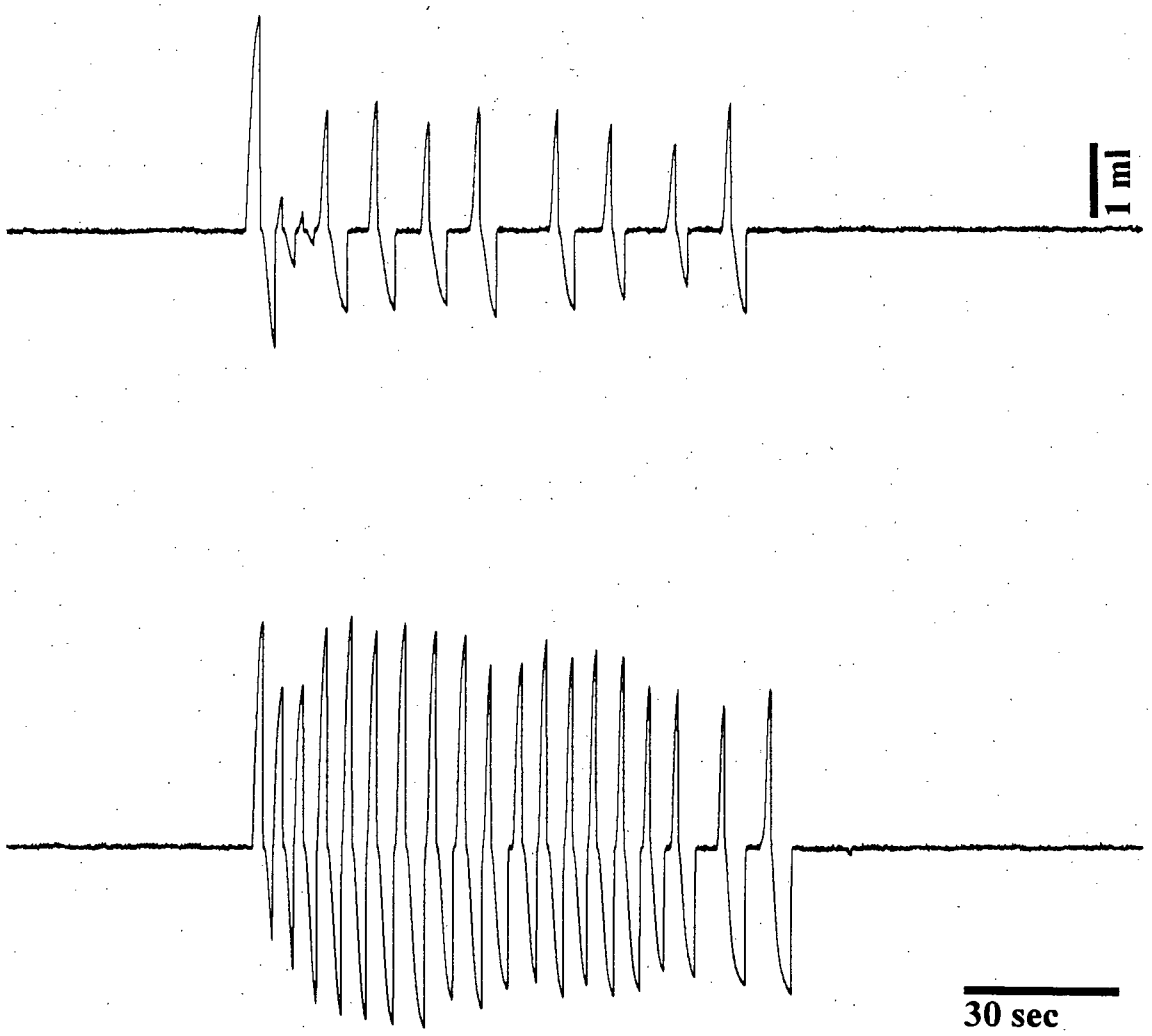

1 ml
30 sec

Fig. 5.7

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E), breaths per episode, episodes per hour and the instantaneous frequency of breathing within episodes in hibernating animals before (open bars) and after (hatched bars) MK-801 treatment. The "*" denotes a significant difference from values before treatment ($P<0.05$).

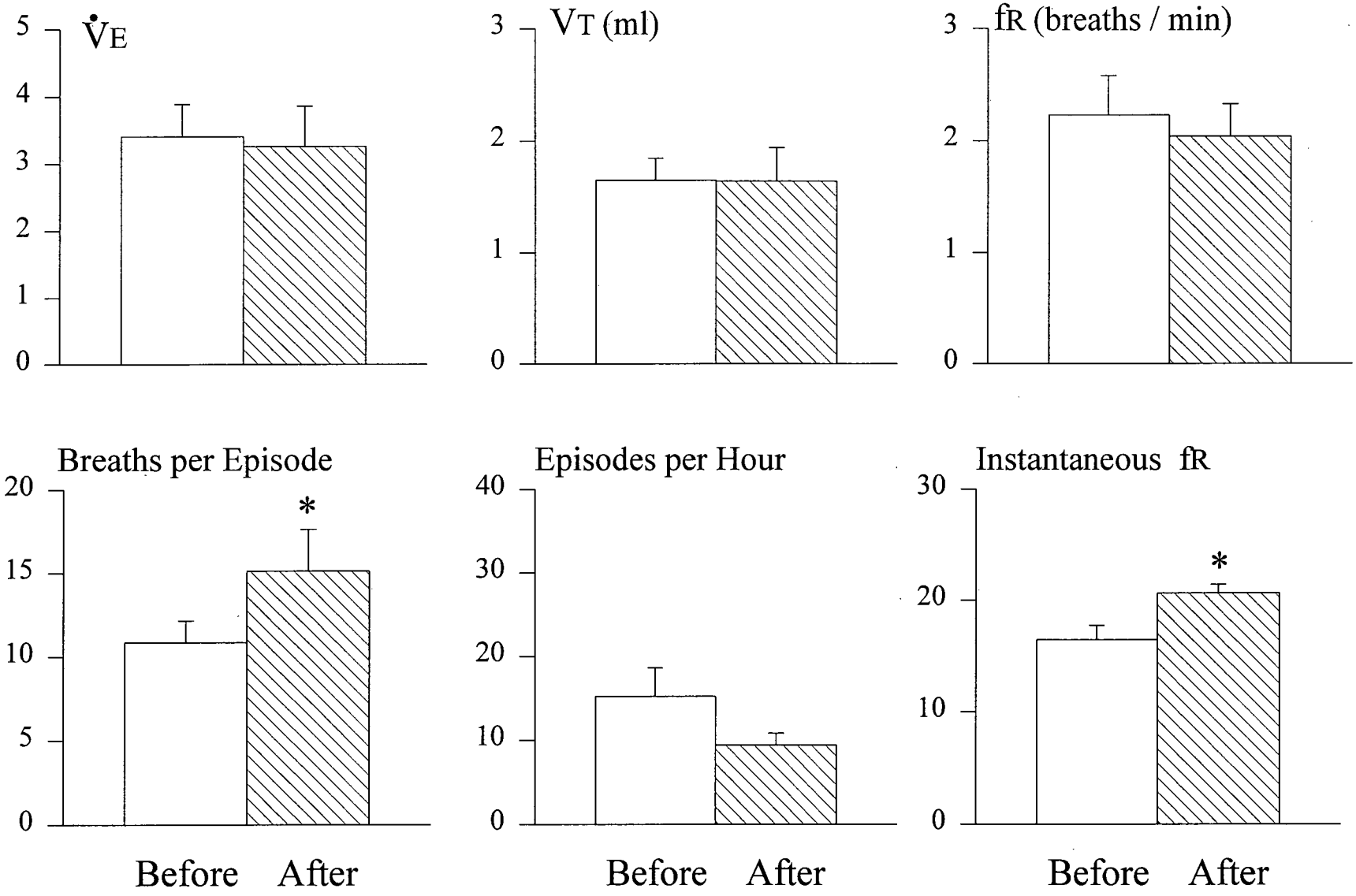
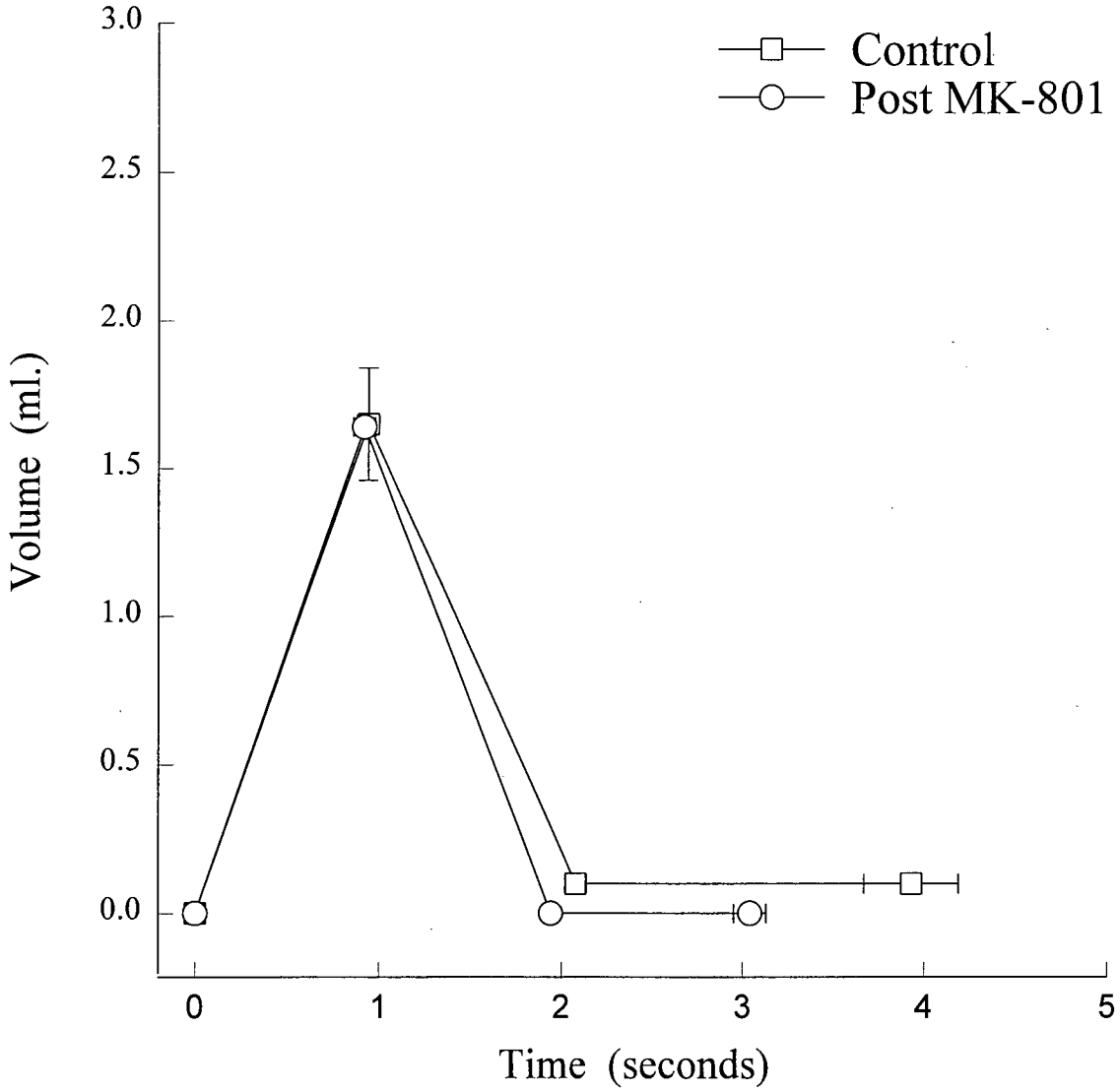

$\dot{V}_E$
5
4
3
2
1
0
V_T (ml)
3
2
1
0
fR (breaths / min)
3
2
1
0
Breaths per Episode
20
15
10
5
0
*
Before
After
Episodes per Hour
40
30
20
10
0
Before
After
Instantaneous fR
30
20
10
0
*
Before
After

Fig. 5.8

Representation of mean (± standard error) inspiratory volumes and durations of inspiration and expiration of hibernating animals before (□) and after (○) MK-801 treatment. Statistical differences are noted in the text.


Control
Post MK-801
Volume (ml.)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Time (seconds)

5.4. Discussion

Investigations of numerous mammal species have shown that disruption of the function of NMDA receptors through systemic application of MK-801 has a similar influence on breathing pattern to that produced by lesion or ablation of the PBC, and have shown that microinjection of MK-801 directly into the PBC has the same influence as general administration of this drug (Ling et al. 1994; Wang et al. 1993; Jhamandas and Harris, 1992). As general administration of MK-801 will antagonize NMDA receptors throughout the CNS, these investigations suggest that the only NMDA receptors involved in the control of resting breathing pattern are located within the PBC (Ling et al. 1994; Fung et al. 1994; Cassus-Soulanis et al. 1995; Bonham, 1995 q.v.). The distribution of NMDA receptors in the CNS of ground squirrels is, at present, unknown and a detailed analysis of this is currently underway. In the present study, the assumption is made that the distribution of NMDA receptor function and the influences of MK-801 treatment are the same as in other mammalian species studied to date, ie, in terms of ventilatory control, MK-801 primarily interrupts NMDA receptor-mediated processes within the PBC.

5.4.1 Euthermic animals

5.4.1.1 Arousal state

MK-801 administration abolished the expression of SWS and resulted in a continuous state of QW. It is believed that wakefulness results from the activation of the thalamus and cortex by brainstem nuclei comprising the midbrain reticular formation, and that sleep occurs in the absence of stimulation from this "reticular activating system" (RAS) (Steriade, 1996 q.v.). These observations suggest that the expression of SWS, (or the production of an EEG pattern

indicative of SWS) is reliant on an NMDA receptor-mediated glutamatergic process which is involved in the inhibition of this activation pathway at some point. This inhibition is interrupted by NMDA receptor antagonism, resulting in a constant state of higher brain activation and a constant period of QW.

Activation of the NMDA receptor, under permissive conditions, results in Ca^{2+} and Na^{+} influx, and either membrane depolarization and the generation of action potentials or the activation of Ca^{2+}-mediated second-messenger systems (Mayer and Miller, 1991). The MK-801 molecule enters and blocks the open ion channel associated with the NMDA receptor, rather than competing for the binding site for glutamate itself. Thus, MK-801 acts as a noncompetitive antagonist to the NMDA receptor, preventing the activity of glutamatergic excitation that would otherwise occur (Collingridge and Singer, 1991; Huettner and Bean, 1988). The results of MK-801 treatment are, thus, attributed to deactivation or the removal of excitatory influences rather than direct inhibition. MK-801 treatment can deactivate either excitatory or inhibitory neurons and, thus, this treatment can result in either a net inhibition or excitation, ie. a net negative or positive modulation resulting from the removal of either excitatory or inhibitory influences respectively.

In support of the observation that MK-801 treatment produces a constant state of QW, stimulation of portions of the lateral or anterior hypothalamus, or the thalamic portion of the RAS can inhibit the mesencephalic portion, causing somnolence and sleep (Leach et al. 1980; Parmeggiani and Morrison, 1990 q.v.). In addition, stimulation of serotonergic cells in the raphe nuclei produces sleep and deactivation of these cells prevents it, and lesions in the raphe nuclei or the anterior hypothalamus produce wakefulness by removing the inhibition of excitatory reticular nuclei (Parmeggiani and Morrison, 1990 q.v.; Riekkinen et al. 1991;

Steriade, 1992). Immunocytochemical surveys (Petralia et al. 1994) indicate the presence of NMDA receptors within all of these areas which could underlie the observations of the present investigation.

In contrast to these observations, MK-801 has been demonstrated to directly induce an intoxicated or somnolent state, with reduced activity and responsiveness, and a SWS-like EEG when administered systemically in unanesthetized rodents (Foutz et al. 1988; Wishaw and Auer, 1989; Wozniak et al. 1990; Marquis et al. 1989). It has also been shown to induce a period of intoxication distinct from sleep, but accompanied by cortical EEG with increased amplitude and sharp slow waves, followed by a period of intense and prolonged sleep (Campbell and Feinberg, 1996). These studies suggest that NMDA receptor-mediated mechanisms are involved in the prevention of somnolence such that blockade of these processes by MK-801 allows the somnolence to be expressed. This conclusion would seem to be appropriate as MK-801 targets the ion channel associated with the NMDA receptor in the same manner as the dissociative anesthetics ketamine, and pencyclidine which induce sedation, immobility and marked changes in EEG (Lodge and Johnson, 1991; Marshall and Wollman, 1980).

The induction of somnolence and SWS-like EEG by NMDA antagonism is not, however, a universal observation. Bissonnette et al. (1993) have demonstrated that competitive blockade of the NMDA receptor produced prolonged periods of behavioural "arousal" in unanesthetized fetal sheep. These authors have shown that noncompetitive blockade by MK-801 in unanesthetized fetal sheep greatly enhanced the expression of low-voltage electrocortical activity (lv-ECoG). This lv-ECoG state was associated with tonic or phasic nuchal muscle activity and resembled a naturally occurring fetal behavioral state considered analogous to

wakefulness. This same treatment resulted in the reduced occurrences of either periods of high-voltage electrocortical activity or lv-ECoG associated with nuchal muscle atonia, characteristic of the fetal analogues to slow-wave and REM sleep respectively (Bissonnette et al. 1995). Thus, the influences of NMDA-type glutamatergic blockade on adult *S. lateralis* are similar to those of prenatal mammals rather than the typical reaction exhibited by adult rodents

A parallel has been drawn between mammals such as *S. lateralis*, which exhibit seasonal hibernation, and neonatal mammals (Pearson and Greenway, 1990). There is a body of evidence that suggests that mammals capable of hibernation exhibit many traits normally associated with prenatal and neonatal animals, such as a high proportion of body fat, the retention of brown adipose tissue and high pulmonary compliance (Pearson and Greenway, 1990; Hayward and Ball, 1966; Milsom and Reid, 1995). In support of this, the results outlined in chapter 3 document that adult ground squirrels exhibit a dependance on feedback from pulmonary receptors which resembles that of neonatal rats but is unlike that of other adult rodents. It may be that the ability to hibernate is a neotenous characteristic, and that animals which have developed the ability to hibernate have done so by retaining juvenile characteristics into adulthood. Thus, this seemingly uncommon induction of constant wakefulness by MK-801 treatment observed in the squirrels may simply be the retention of a characteristic not otherwise observed in adult mammals which do not hibernate.

In general, dissociative anesthetics such as ketamine act at the level of the cortex and limbic systems, rather than the reticular system (Lodge and Johnson, 1991; Marshall and Wollman, 1980; Winters et al. 1972), while the reticular system is responsible for mediating the activation of higher centers producing transitions between states of sleep and wakefulness (Steriade , 1996 q.v.). Glutamate has been shown to be one of the main neurotransmitters used

by the reticular activating system (Steriade , 1996 q.v.), and autoradiographic and immunocytochemical surveys in the rat have shown that the reticular system, particularly the midbrain reticular formation and its component nuclei contain high concentrations of NMDA receptors (Petralia et al. 1994). Given the importance of these areas in the genesis and stability of wakefulness, this population of receptors is at least one likely site where blockade may be responsible for the constant QW-like EEG pattern observed following MK-810, although the precise location of the neural area responsible for this observation is not within the scope of this investigation.

5.4.1.2. Ventilation

Treatment with MK-801 did not alter any aspect of breathing that could not be attributed to the switch from SWS to QW. Orem (1994) proposed that wakefulness provides an "arousal" stimulus to breathe, which elevates breathing to levels above those observed during sleep. As MK-801 treatment induced a constant state of QW, such treatment could indirectly produce a tonic "arousal" stimulus which maintains breathing at the levels associated with QW.

Chapman (et al. 1982) proposed that there is a tonic source of inhibition acting on the ventilatory control system which limits breathing frequency. Thus, breathing during sleep could be actively depressed through a process involving glutamatergic activation of NMDA receptors. In this case, MK-801 treatment would result in a direct dis-inhibition of breathing, and breathing would return to the levels associated with QW.

There is little constancy in the ventilatory responses to this treatment documented in the literature for other species. Although NMDA receptor antagonism by MK-801 has been

reported to depress overall ventilation in anesthetized dogs, cats and rats through decreases in f_R and/or V_T (McManigle et al. 1994; Abrahams, et al. 1993; Ang et al. 1992; Connelly et al. 1992), unanesthetized mice and pigs have been shown to increase f_R following MK-801 treatment, while unanesthetized cats exhibit a decrease in f_R, and unanesthetized guinea pigs and rats show no change in breathing (Foutz et al. 1988; Loscher et al. 1991; Cassus-Soulanis et al. 1995; Morrison, 1996). The unanesthetized in vitro guinea pig brainstem-spinal cord preparation exhibits a dose-dependent increase in f_R following MK-801 administration (Morin-Surun et al. 1995), while the same preparation using rats does not (Greer et al. 1991). There is, however, preliminary evidence to suggest that anesthetized Sprague-Dawley rats also exhibit an increase in f_R following administration of MK-801 into cerebral-spinal fluid in the same doses as those outlined in the present investigation (Boon and Milsom, unpublished).

5.4.2 Anesthetized animals

5.4.2.1. Administration into csf

Administration of MK-801 directly into the cerebral-spinal fluid, rather than into the peripheral circulation resulted in a continuous State I pattern of EEG and stimulation of f_R and V_E. This result is similar to that which occurred following systemic application of MK-801. Thus, the differences observed between unanesthetized animals which received MK-801 directly into the cerebral-spinal fluid and anesthetized animals which received this drug in the peripheral circulation are due to differences between the anesthetized and unanesthetized groups, rather than to differential influences of rout of drug infusion.

5.4.2.2 Arousal state

Administration of MK-801 into the peripheral circulation resulted in a consistent State-I type EEG which persisted for the duration of the experiment. This observation again suggests that the mechanisms directly responsible for the expression of State-III during urethane anesthesia are reliant on an NMDA receptor-mediated glutamatergic process which is interrupted by NMDA receptor antagonism. Alternately, the "activation" occurring during State I could normally be inhibited during State III, via an NMDA receptor-mediated process and this inhibition could be removed by NMDA receptor antagonism. This observation compliments that made on unanesthetized animals, and contrasts with reports of MK-801-induced intoxication and somnolence in other adult animals (Foutz et al. 1988; Wishaw and Auer, 1989; Wozniak et al. 1990; Marquis et al. 1989; Campbell and Feinberg, 1996; Lin et al. 1996).

5.4.2.3. Ventilation

Treatment with MK-801 induced significant changes in breathing pattern, manifested as increases in f_R and $\dot{V}_E$. The increase in f_R was produced by a decrease in both T_{EA} and T_{EP}. This suggests that an NMDA receptor-mediated glutamatergic process tonically inhibits breathing frequency and acts to maintain the pause between breaths. In the absence of this inhibition, the pause between breaths decreases and f_R increases. This pattern of change is identical to that seen in the unanesthetized animals, with one notable difference. Levels of f_R, V_T and $\dot{V}_E$ are all considerably lower during anesthesia than in unanesthetized animals, reflecting a significant ventilatory depression induced by anesthesia. NMDA receptor antagonism during anesthesia results in a constant State I EEG and an elevation of f_R to levels

surpassing those observed during State I, yet similar to those observed during QW.

There are two possible sources of ventilatory inhibition at work; one which depresses f_R between State I and State III, and another which depresses f_R, V_T and $\dot{V}_E$ between unanesthetized and anesthetized conditions. To explain the differences between States I and III, the same "arousal" stimulus to breath, which elevates breathing during wakefulness above levels observed during sleep (Orem, 1994) could be responsible for the elevation of breathing during state I above levels observed during state III during the anesthesia. This pattern may result from the same type of direct or indirect inhibition of breathing during State III that was proposed to occur during SWS. The elevation in f_R associated with MK-801 treatment, however, far exceeds the slight changes in f_R which occur between State I and III. Given that MK-801 blocks otherwise active processes, these results indicate that f_R is depressed between unanesthetized QW and anesthetized States I through an active inhibition, which is facilitated by glutamatergic activation of NMDA receptors. MK-801 treatment results in a disinhibition of f_R which then returns to unanesthetized QW levels. V_T is still lower than in unanesthetized animals and $\dot{V}_E$, although significantly elevated by MK-801 treatment, is still well below that observed in unanesthetized animals. Therefore, the depression of V_T during anesthesia is not governed by NMDA receptor-mediated processes, while the depression of f_R appears to be.

5.4.3 Hibernating animals

Antagonism of NMDA receptors resulted in an increase in the frequency with which breaths were taken within each breathing episode, and in the number of breaths that were taken in each of these episodes. Like the results obtained from anesthetized animals, the data from hibernating animals are consistent with the theory that there is a tonic source of inhibition

acting on the ventilatory control system which limits breathing frequency, and that this inhibition is facilitated by an NMDA receptor-mediated process. Such NMDA receptor-mediated processes are not, however, involved in the initiation and termination of breathing episodes themselves, as these persist following MK-801 treatment. Thus, NMDA receptor antagonism results in a disinhibition of breathing frequency which is manifest only during the episodes when breathing occurs. There must also be a second source of control which either stimulates breathing to produce the episodes, or inhibits breathing during the apnea.

In some animals, MK-801 treatment was followed by arousal from hibernation. Alterations in breathing pattern following MK-801 treatment occurred regardless of whether animals aroused from hibernation or not. In the cases where arousal was stimulated, changes in breathing pattern occurred well before typical signs of arousal, such as tachycardia and shivering, were evident. It appears, therefore, that the changes in breathing pattern were due to a direct influence of the drug treatment, rather than an indirect result of the stimulation of arousal. The observation that MK-801 stimulated arousal from hibernation in some animals is interesting, however, and suggest that the hibernation state, per se, may be facilitated by the activation of an NMDA receptor-mediated process (Harris and Milsom, in preparation).

5.5. Conclusion

Taken together, the results from the unanesthetized and anesthetized euthermic animals indicate that the "arousal stimulus" to breathe, present during wakefulness, or the wake-like state of urethane anesthesia, appears to be due to removal of a mild inhibition of f_R during SWS or the sleep-like state. This inhibition is facilitated by glutamatergic activation of NMDA receptors, and is removed by MK-801 treatment. Anesthesia itself produces a significant degree of respiratory inhibition, resulting in a reduction of f_R and V_T. MK-801 treatment elevated f_R (but not V_T) to levels similar to those observed during QW. This suggests that the inhibition of f_R by urethane anesthesia is also facilitated by activation of NMDA receptors. Finally, during hibernation, MK-801 treatment resulted in an elevation of instantaneous breathing frequency within breathing episodes and an increase in the number of breaths in each episode. This suggests that there is an active prolongation of the pause between individual breaths in each breathing episode which is facilitated by the activity of NMDA receptors. The episodes themselves persist following MK-801 treatment and their presence is, thus, not reliant on a glutamatergic process.

Chapter 6

The Influence of NMDA Receptor-Mediated Processes on Ventilatory Chemoresponses in Ground Squirrels

Abstract

The effects of blockade of the function of N-methyl-D-aspartate (NMDA) type glutamate receptors by the non-competitive antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801) on ventilatory responses to hypoxia and hypercapnia were assessed in the golden-mantled ground squirrel, *Spermophilis lateralis,* without anesthesia and during anesthesia, and natural hibernation.

Hypoxia (10 % O_2 in N_2) increased ventilation in unanesthetized and anesthetized squirrels primarily through increases in breathing frequency while hypercapnia (5% CO_2 in air) increased ventilation primarily through increases in tidal volume. MK-801 treatment did not alter resting breathing pattern in unanesthetized animals. This treatment did not alter overall levels of ventilation but did change breathing frequency and tidal volume components of the hypercapnic ventilatory response. The ventilatory response to hypoxia, however, was suppressed. Treatment with MK-801 in anesthetized animals stimulated ventilation and elevated breathing frequency to levels observed without anesthesia. Again, however, the hypoxic ventilatory response was suppressed following MK-801 treatment while the hypercapnic response, although dampened, was retained.

During hibernation, animals breathed episodically. They exhibited a robust ventilatory response to hypercapnia primarily due to an increase in the length of breathing episodes, but did not respond to breathing a 7% O_2 in N_2 gas mixture. MK-801 treatment increased the number of breaths in each breathing episode and the frequency of breathing during these episodes, but did not alter overall ventilation. Hypercapnia, following MK-801 treatment, stimulated ventilation through increases in the number of breaths per breathing episode and the frequency of episodes, while hypoxia still had no influence.

The data indicate that antagonism of NMDA receptors with MK-801 suppressed the hypoxic ventilatory response and, thus, this response must be facilitated by glutamatergic activation of NMDA receptors. The hypercapnic ventilatory response persisted despite MK-801 treatment, indicating that this response is not dependent on NMDA receptors-mediated processes.

6.1. Introduction

In chapter 5, it was demonstrated that ventilation in awake euthermic golden-mantled ground squirrels was not influenced by functional blockade of N-methyl-D-aspartate (NMDA) type glutamate receptors by the non-competitive antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801). Following this treatment, however, animals no longer exhibited sleep. In anesthetized animals, ventilation was stimulated by MK-801 treatment and these animals, too, no longer exhibited the slow-wave-sleep-like state normally observed during anesthesia. These data indicated that NMDA receptors were important for the expression of sleep and sleep-like states of anesthesia, and for the depression of breathing frequency observed both with sleep, and anesthesia. In hibernating animals, while MK-801 treatment did not alter overall ventilation it elevated both the number of breaths in each breathing episode, and the frequency with which these breaths occurred within the episode. Under these conditions, NMDA receptor-mediated processes appear important for the regulation of breathing pattern and act to reduce breathing within episodes.

Glutamate, acting via NMDA receptors, facilitates the ventilatory response to hypoxia and treatment with NMDA antagonists attenuates or blocks this response (Kubo et al. 1993; Soto-Arape et al. 1995; Lin et al. 1996; Ang et al. 1992; Chae et al. 1993; Miyawaki et al. 1994). These studies indicated that NMDA receptor antagonism interrupted the integration of afferent input from the carotid body chemoreceptors, transmitted via the carotid sinus nerve, at the level of the nucleus tractus solitarii (NTS) (Kubo et al. 1993; Vardhan et al. 1993; Ogawa et al. 1995; Mizusawa et al. 1994; Haxhiu et al. 1995), the phrenic nucleus (Chitravanshi and Sapru, 1997), and the rostroventrolateral reticular nucleus (Sun and Reis, 1995).

Dogs and rats both respond to hypoxia by increasing both breathing frequency (f_R) and

tidal volume (V_T). Following MK-801 treatment these animals still exhibit an hypoxic ventilatory response but it is dampened, primarily because V_T no longer changes, while changes in f_R persist (Ang et al. 1992; Mizusawa et al. 1994). These results indicate that MK-801 treatment does not interfere with the transduction of the input from carotid body chemoreceptors themselves but, rather, it interferes with the integration of this information and its effects on tidal volume regulation.

NMDA receptor-mediated processes also contribute to the ventilatory response to hypercapnia. Antagonism of a population of these receptors located proximal to the surface of the ventrolareral medulla, within the retrotrapezoid nucleus, decreases the amplitude of phrenic neurogram responses to CO_2 (Nattie et al. 1993, Nattie and Li, 1995). The ventilatory response to hypercapnia is modulated by an area of the dorsal pons, consisting of the lateral and medial Parabrachial and Kölliker-Fuse nuclei, referred to as the Parabrachial complex (PBC). Ablation of this area decreases the magnitude of hypercapnic ventilatory responses by removing the f_R component of this response (St.John, 1977). More recent studies have indicated that the PBC contains a high proportion of NMDA receptors (Monaghan and Cotman, 1985; Petralia et al. 1994). Thus, pathways utilizing these receptors could be involved in modulation of the f_R component of the hypercapnic ventilatory response by the PBC, although this possibility has not been assessed.

Thus, in species studied to date, MK-801 treatment eliminates the changes in V_T associated with hypoxia, and ablation of the PBC eliminates the change in f_R associated with hypercapnia. Ground squirrels whether anesthetized or unanesthetized, however, primarily increase f_R in response to hypoxia, and increase V_T in response to hypercapnia (chapter 3). Thus, it would be predicted that MK-801 treatment should have little or no influence on the

hypoxic and hypercapnic ventilatory responses of both groups of ground squirrels.

In hibernating squirrels, MK-801 treatment has no effect on overall ventilation but does alter breathing pattern by increasing the number of breaths per breathing episode and the frequency of breathing within episodes (chapter 5). Hibernating animals respond to hypoxia with a modest decrease in the number of breaths per episode and increase in the frequency of episodes, and respond to hypercapnia with a robust increase in both the number of breaths per episode and frequency of episodes (Webb and Milsom, 1994; Harris and Milsom, 1995, chapter 4). It would be predicted, in this case, that MK-801 treatment should alter the ventilatory responses to changes in O_2 and CO_2.

This study was designed to test these predictions by comparing the ventilatory responses of awake, anesthetized, and hibernating squirrels to hypoxia and hypercapnia before and after MK-801 treatment.

6.2. Methods

6.2.1. Instrumentation

Experiments were performed on the same ground squirrels *(Spermophilus lateralis)* described in chapter 5. Animals used in the chronic studies were surgically instrumented *a priori.* Animals were fitted with four electroencephalographic (EEG), two electrocardiographic (ECG), two electromyographic (EMG) and two respiratory impedance electrodes, and a third cerebral ventricle cannula (Boswell et al. 1993) as outlined in the general methods section (chapter 2).

6.2.1.2. Euthermic animals (chronic study)

Five squirrels selected for this portion of the study underwent surgery during the summer and were held for at least 3 weeks following surgery before experimentation.

Unanesthetized animals were transferred to a whole body plethysmograph. The "dummy" cannula was replaced with a 23-gauge "injection" cannula (Plastics One , Roanoke VA.) which was connected to a 10 µl Hamilton syringe via a length of polyethylene tubing (PE 20). The syringe and tubing were filled with a solution of the non-competitive NMDA-type glutamatergic antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801, Research Biochemicals) in saline (5 mg/ml, 15 mM; pH 7.3 ±0.1) except for the final 2 µl at the tip which was filled with saline, separated from the MK-801 solution by a small (<0.2 µl) air bubble.

Animals were supplied with air and monitored for at least 2 hours. They were then randomly exposed to hypercapnic (5% CO_2 in air) and hypoxic (10% O_2 in nitrogen) gas mixtures for at least 30 minute periods separated by 30 minutes of exposure to air. Animals

were then monitored for 30 minutes while exposed to air following a 2 µl injection of saline (sham) into the third ventricle, and for 30 minutes following a 2 µl injection of MK-801 solution. Animals were again randomly exposed to hypercapnic and hypoxic gas mixtures for 30 minute periods separated by 30 minutes of exposure to air. Additional injections of MK-801 solution were repeated every 30 to 45 minutes.

6.2.1.3. *Hibernating animals*

Twelve animals selected for this portion of the study underwent surgery during the winter. Following recovery, these animals reentered hibernation. During deep hibernation, animals were transferred to a plexiglass box (12 x 20 x 10 cm) within a laboratory controlled-environment chamber. The box was supplied with air at a flow rate of 500 ml per minute. Ambient temperature was held at 5 ± 1 °C. Animals were fitted with a mask connected to a resistance pneumotachograph to measure ventilation. The "dummy" cannula was replaced with a 23-gauge "injection" cannula (Plastics One , Roanoke VA.) which was connected to a 10 µl Hamilton syringe as described above.

Animal were monitored; i) for at least two hours to provide initial control values, ii) for at least two hours while the test box was supplied with hypercapnic (5% CO_2 in air) gas, iii) for at least two hours while the test box was supplied with hypoxic (7% O_2 in N_2) gas, iv) for at least one hour during exposure to air following a 2 µl injection of saline (sham treatment) into the third ventricle, v) for at least one hour following a 2 µl injection of MK-801 solution, and vi) again during 2 hour periods of exposure to hypercapnic and hypoxic gases. Supplemental (2 µl) injections of MK-801 solution were made every hour during this procedure.

6.2.1.4. *Anesthetized animals (acute study)*

Seven ground squirrels were used in this series of experiments. Animals were anesthetized with urethane, tracheotomized, fitted with EEG, ECG, EMG and respiratory impedance electrodes and had catheters implanted in a femoral artery and vein, as described in chapter 2.

Animals were supplied with air and monitored; i) for at least two hours to provide initial control values, ii) during random exposure to hypercapnic (5% CO_2 in air) and hypoxic (10% O_2 in nitrogen) gas mixtures for at least 30 minute periods separated by 30 minutes of exposure to air, iii) for at least one hour following a 0.3 ml intravenous infusion of saline (sham) into the venous catheter, iv) for one hour following a 0.2 to 0.3 ml infusion of a 0.15 mg/ml solution of MK-801 in saline (pH 7.3 ±0.1) resulting in a dose of 0.15 - 0.30 mg MK-801 per kg body mass and, v) again following exposure to hypoxic and hypercapnic gas mixtures. Additional 0.2 to 0.3 ml infusions of MK-801 solution were administered every hour.

6.2.1.5. *Additional anesthetized animals*

Anesthetized animals in the aforementioned study received MK-801 through intravenous injection, while those used in the unanesthetized studies received MK-801 treatment via direct injection into the cerebrospinal fluid. This drug easily crosses the blood-brain barrier, and is commonly administered by intravenous or intra peritoneal injection (Cassus-Soulanis et al. 1995). To assess the potential difference which route of drug infusion may have had on the results, however, three of the animals instrumented and used in the unanesthetized portion of this series were subsequently tested during anesthesia. These animals

were anesthetized with a 5ml/kg intra peritoneal injection of a 20% solution of Urethane, supplemented with vaporous halothane. The trachea was cannulated, halothane was discontinued and the animals were transferred to the homeothermic table within the electrically shielded chamber. The tracheal catheter was connected to a ventilatory pneumotachograph and air line and the animals left to stabilize for approximately two hours. Arterial and venous catheters were not implanted in these animals, and EEG, EMG, and EKG were monitored using previously implanted electrodes.

Animal were monitored; i) for at least two hours to provide initial control values, ii) during random exposure to hypercapnic (5% CO_2 in air) and hypoxic (10% O_2 in nitrogen) gas mixtures for at least 30 minute periods separated by 30 minutes of exposure to air, iii) for at least one hour following a 2 µl injection of saline (sham treatment) into the third ventricle, iv) for at least one hour following a 2 µl injection of MK-801 solution, and v) again following exposure to hypoxic and hypercapnic gas mixtures. Additional 2 µl injections of MK-801 solution were administered every hour.

6.3. Results

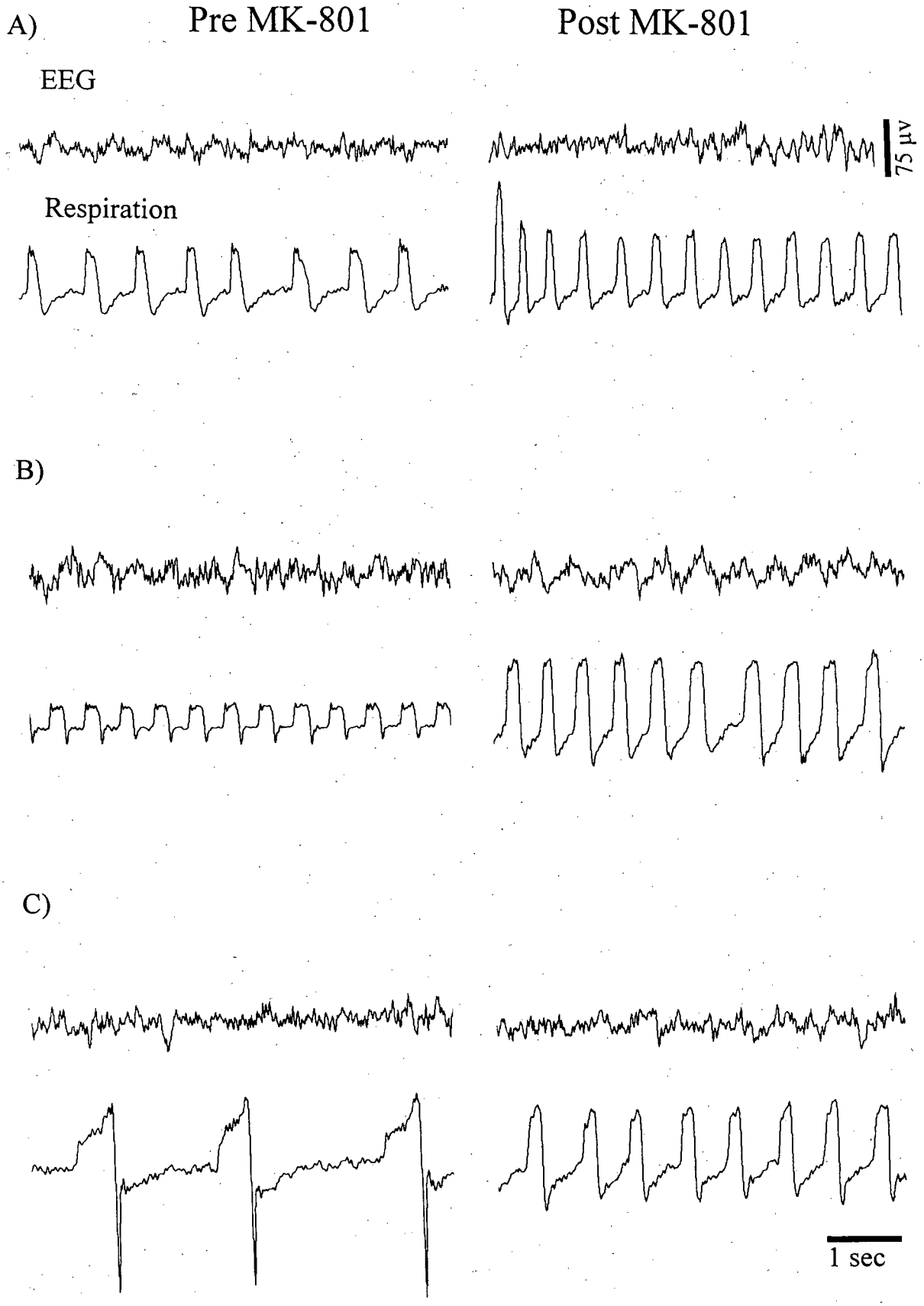
6.3.1. Euthermic animals

6.3.1.1 Control conditions

As it was reported in chapter 5, animals regularly cycled between periods of quiet wakefulness (QW) and slow-wave sleep (SWS), before treatment and following saline injection (sham treatment) but exhibited a constant state of QW following MK-801 treatment. As a result, breathing and ventilatory chemoresponses were characterized and compared only during QW (Fig. 6.1).

Fig. 6.1

Representative recordings of EEG and breathing of an unanesthetized squirrel exposed to A) air, B) hypoxia (10% O_2) and C) hypercapnia (5% CO_2) during quiet wakefulness before (Pre-MK-801) and following MK-801 treatment (Post) Breathing records are from the output of the plethysmograph, with inspiration as an upward deflection and tidal volume proportional to the area under the deflection.


A)
Pre MK-801
Post MK-801
EEG
75 µv
Respiration
B)
C)
1 sec

Before MK-801 treatment, hypoxia produced non-significant increases in both respiratory frequency (f_R) (from 122.0 ± 6.7 to 159.1 ± 29.6 br/min) and tidal volume (V_T) (from 2.14 ± 0.24 to 4.91 ± 0.73 ml), which together resulted in a significant increase in ventilation (V_E) (149%, from 270.3 ± 30.9 to 672.2 ± 52.6 ml/min) (Fig. 6.2). There were no significant changes in the timing components of each breath (Fig. 6.3). Hypercapnia produced a 68% decrease in f_R (from 122.0 ± 6.7 to 38.6 ± 5.4) and a 480% increase in V_T (from 2.14 ± 0.24 to 12.42 ± 1.51), resulting in a 60% increase in V_E (from 270.3 ± 30.9 to 432.4 ± 33.7) (Fig. 6.2). Decreases in f_R resulted from increases in durations of non-ventilatory pauses following expiration (T_{EP}) while increases in V_T resulted from an increase in the duration of inspiration (T_I) with no change in the duration of the period of expiratory flow (T_{EA}) or the mean inspiratory flow rate (Fig. 6.3).

6.3.1.2. Post - MK-801 treatment

Injections of saline into the cerebrospinal fluid had no effect on breathing. Breathing following MK-801 treatment was also not significantly different than that observed during QW (Fig. 6.1). While subsequent hypoxia tended to increase f_R (from 139.2 ± 12.0 to 174.0 ± 18.23 br/min), V_T (from 2.35 ± 0.30 to 3.05 ± 0.51 ml), and V_E (from 333.2 ± 50.7 to 532.2 ± 167.3 ml/min) from levels observed during exposure to air, none of these increases were significant (Fig. 6.2). There were also no significant changes in the timing components of each breath (Fig. 6.3). Hypercapnia produced a non-significant decrease in f_R (14%, from 139.2 ± 12.0 to 119.5 ± 3.7 br/min) and an 83% increase in V_T (from 2.35 ± 0.30 to 4.32 ± 0.21 ml), resulting in a 56% increase in V_E (from 333.2 ± 50.7 to 519.4 ± 36.3 ml/min) (Fig. 6.2). Decreases in f_R resulted from increases in T_{EP} while increases in V_T resulted from an increase in T_I with no

Fig. 6.2

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) during exposure to air, 10% O_2 and 5% CO_2 before (open bars) and following MK-801 treatment (hatched bars). The "*" denotes a significant difference from the Pre-MK-801 value during exposure to air, while the "#" denotes a significant difference from the Pre-MK-801 value during exposure to that gas ($P<0.05$).

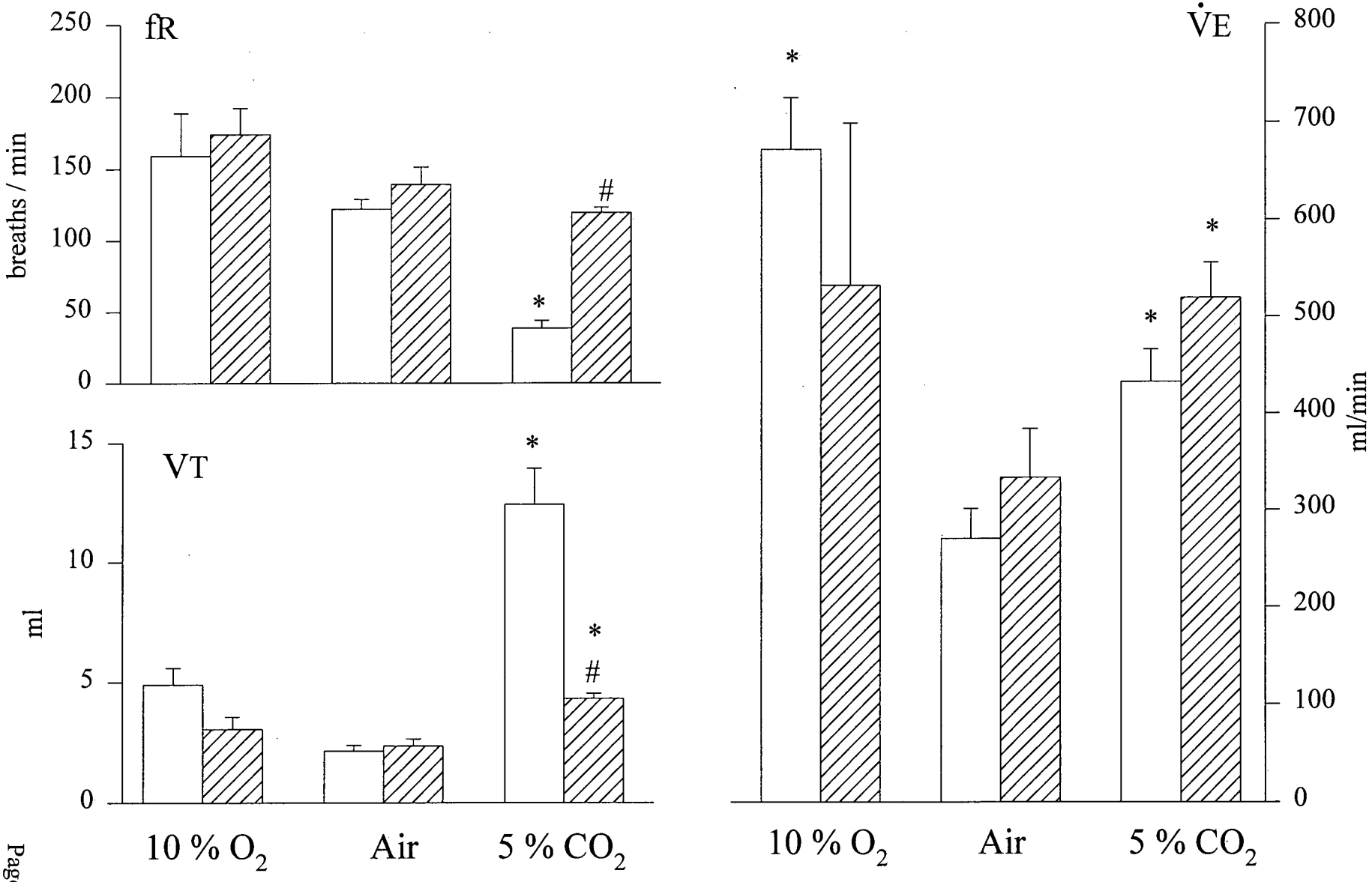
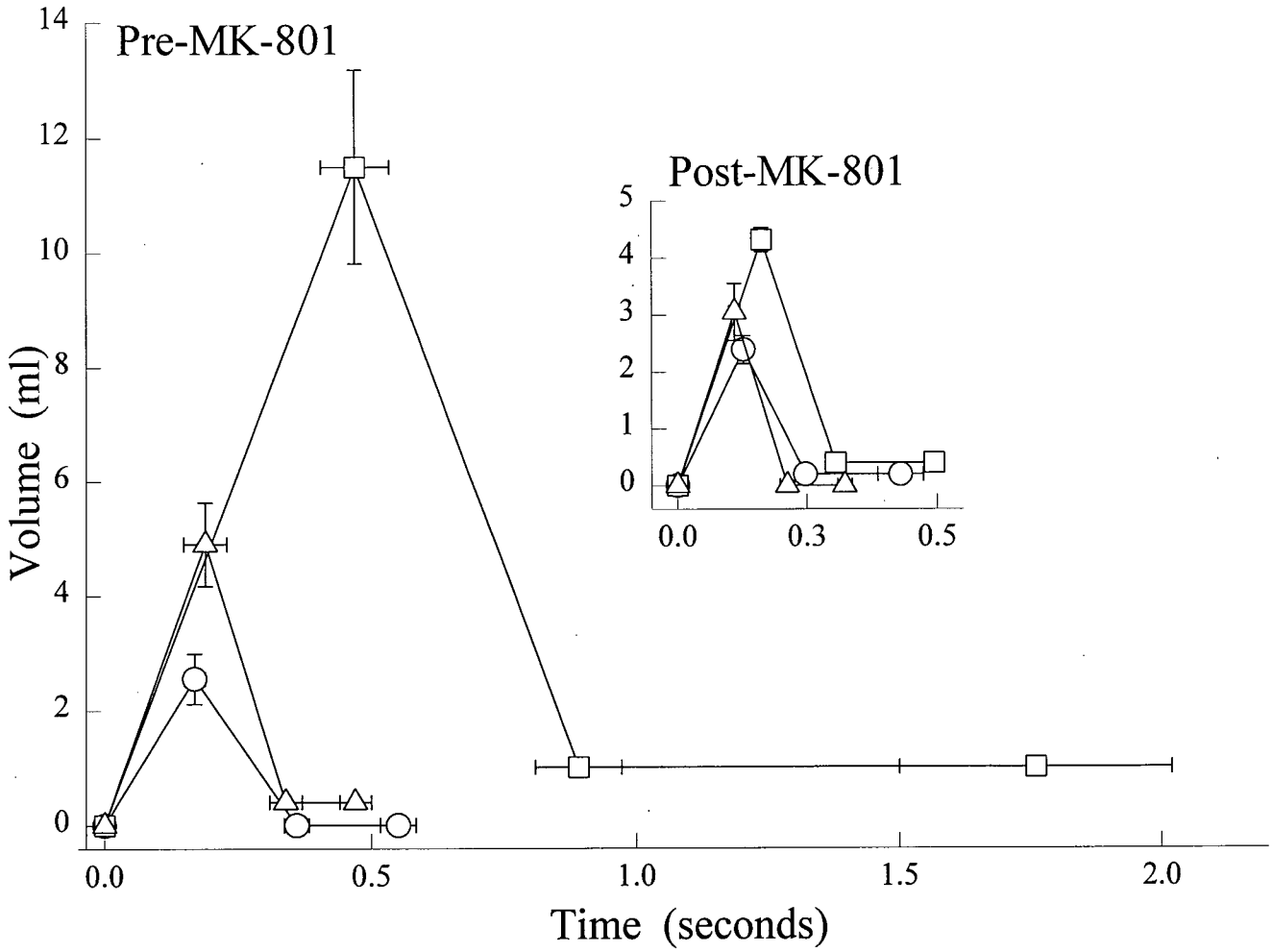

fR
breaths / min
250
200
150
100
50
0
VT
ml
15
10
5
0
VE
ml/min
800
700
600
500
400
300
200
100
0
10 % O2
Air
5 % CO2
10 % O2
Air
5 % CO2

Fig. 6.3

Representation of mean (± standard error) inspiratory volumes and durations of inspiration and expiration, including the end-expiratory pause in unanesthetized animals before (left panel) and following MK-801 treatment (right panel), during exposure to air (○), 10% O_2 (△) and 5% CO_2 (□). Statistical differences are noted in the text. Note that in different plots the end-expiratory volumes have been offset from zero for clarity.


Pre-MK-801
Post-MK-801
Volume (ml)
Time (seconds)
0
2
4
6
8
10
12
14
0.0
0.5
1.0
1.5
2.0
1
3
5
0.3

change in the mean inspiratory flow rate (Fig. 6.3). Additional doses of MK-801 did not alter breathing further.

6.3.2 Anesthetized animals

6.3.2.1. Control conditions

As observed in chapter 5, anesthetized animals regularly cycled between periods of State I (QW-like) and State III (SWS-like), before treatment and following saline injection (sham treatment) but exhibited a constant State I EEG following MK-801 treatment. As a result, breathing and ventilatory chemoresponses were characterized only during State I (Fig.4).

Before MK-801 treatment, hypoxia produced a 113% increase in f_R (from 56.4 ± 4.7 to 119.9 ± 7.8 br/min) and a non-significant decrease in V_T (from 1.33 ± 0.2 to 1.03 ± 0.14 ml), resulting in a 54% increase in V_E (from 71.4 ± 9.0 to 109.8 ± 23.0 ml/min) (Fig. 6.5). Breathing frequency increased due to a decrease in T_{EP} (Fig. 6.6). Hypercapnia produced a 68% increase in f_R (from 56.4 ± 4.7 to 94.6 ± 24.7 br/min) and a 160% increase in V_T (from 1.33 ± 0.20 to 3.47 ± 0.52 ml), resulting in a 333% increase in V_E (from 71.4 ± 9.0 to 309.5 ± 67.5 ml/min) (Fig. 6.5). Breathing frequency was increased by a decrease in T_{EP} while V_T was increased by an increase in mean inspiratory flow rate (Fig. 6.6).

6.3.2.2. Post - MK-801 treatment (intra-venous)

Intravenous injections of saline had no effect on breathing. Following MK-801 injection, however, breathing pattern was different from that observed during State I before the injection. Specifically, there was an 138% increase in f_R resulting in a 116 % increase in overall V_E (Fig. 6.4, 6.5). Subsequent to MK-801 treatment, hypoxia did not alter either f_R

Fig. 6.4

Representative recordings of EEG and breathing of an anesthetized squirrel exposed to A) air, B) hypoxia (10% O_2) and C) hypercapnia (5% CO_2.) during State I before (Pre-MK-801) and following (Post-MK-801) MK-801 treatment. Breathing records are from the integrated output of the pneumotachograph, with inspiration as an upward deflection and tidal volume proportional to the height of the deflection.

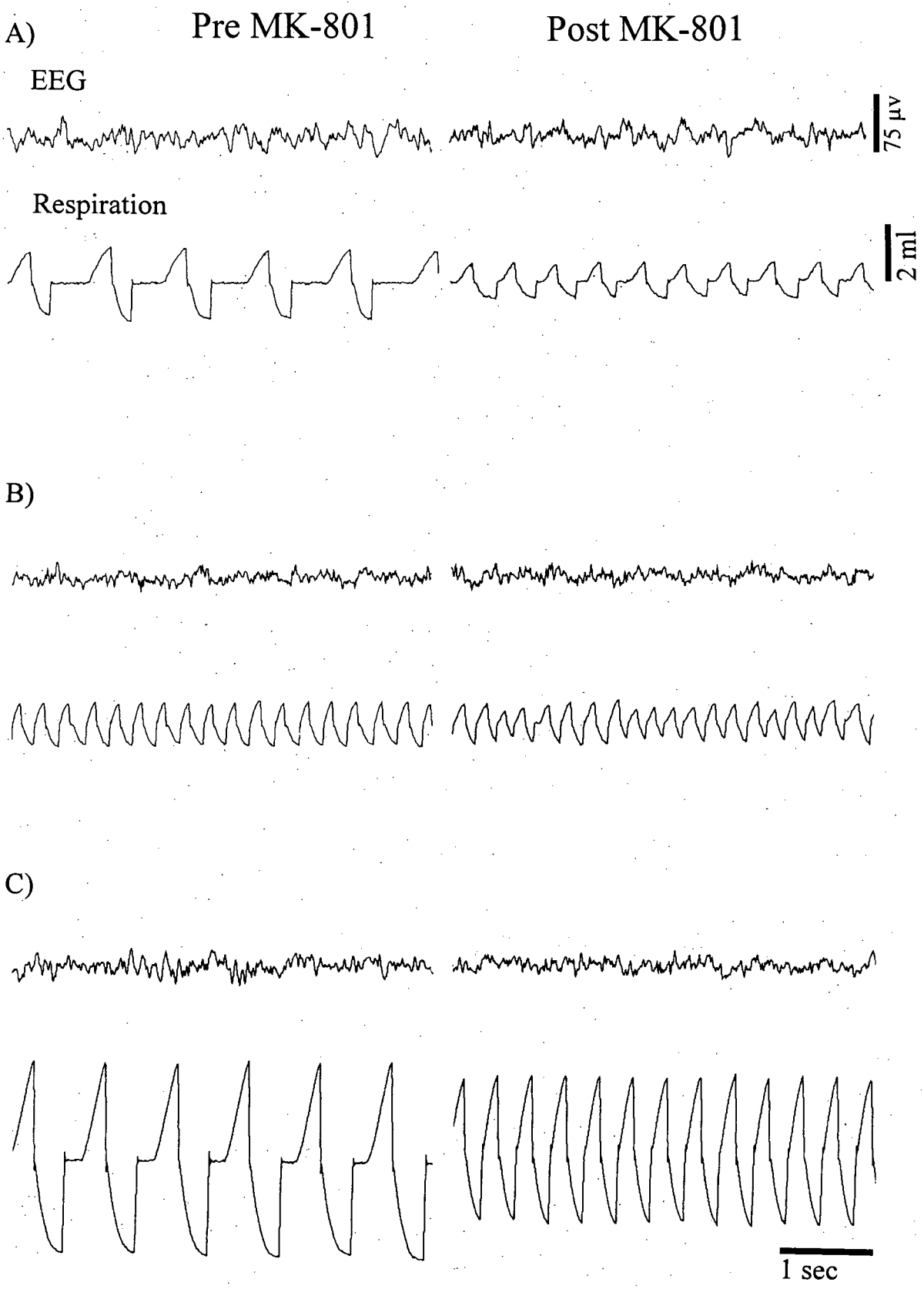

A)
Pre MK-801
Post MK-801
EEG
75 µv
Respiration
2 ml
B)
C)
1 sec

Fig. 6.5

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) of anesthetized animals in before (open bars), and following MK-801 treatment (hatched bars), during exposure to air, 10% O_2 and 5% CO_2. The "*" denotes a significant difference from the air value during pre-MK-801, while the "#" denotes a significant difference from the Pre-MK-801 value during exposure to that gas ($P<0.05$).

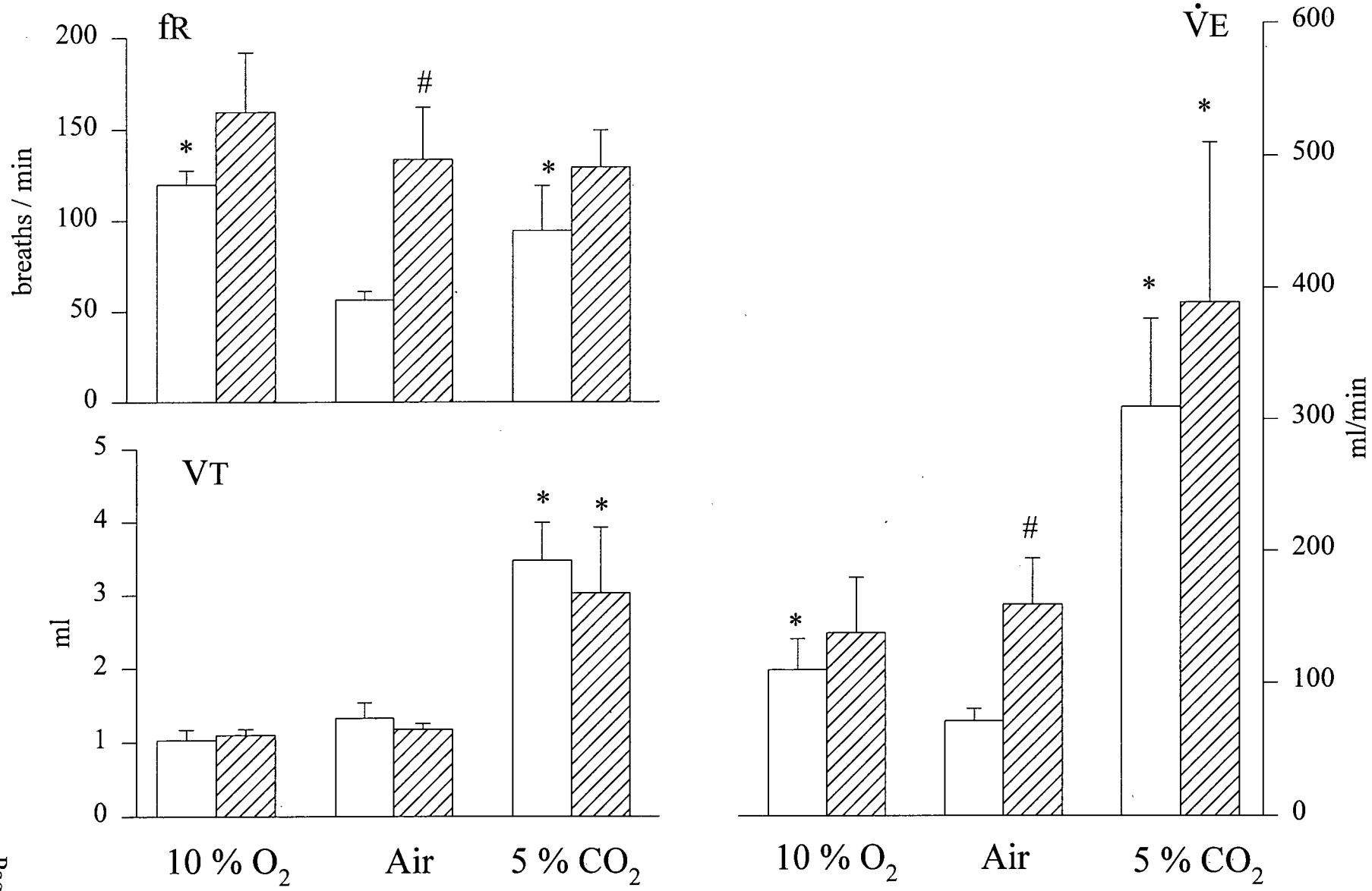
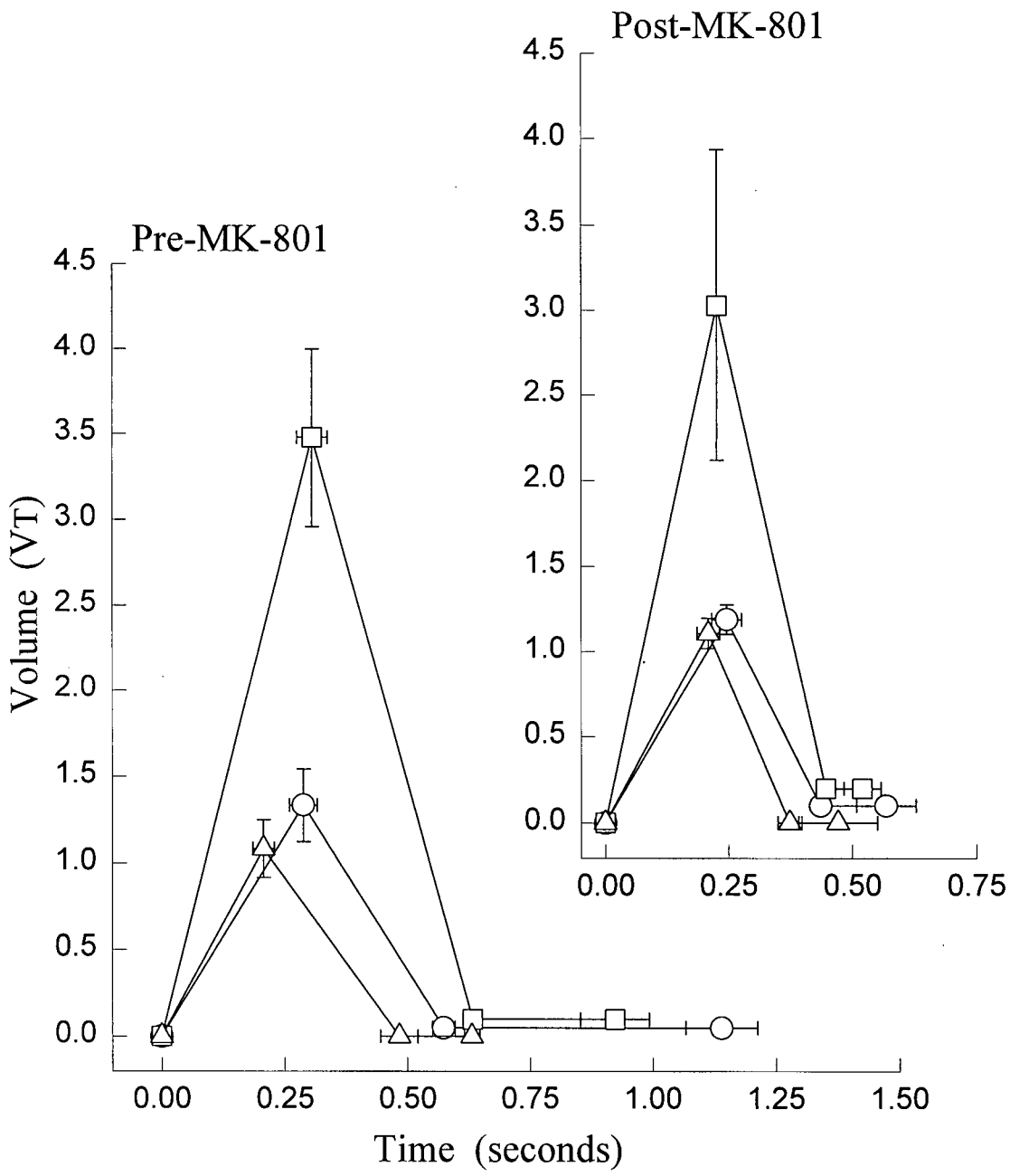

fR
breaths / min
200
150
100
50
0
VT
ml
5
4
3
2
1
0
VE
ml/min
600
500
400
300
200
100
0
10 % O2
Air
5 % CO2
10 % O2
Air
5 % CO2

Fig. 6.6

Representation of mean (± standard error) inspiratory volumes and durations of inspiration and expiration, including the end-expiratory pause in anesthetized animals in State I before (left panel) and following (right panel) MK-801 treatment, during exposure to air (○), 10% O_2 (△) and 5% CO_2 (□). Statistical differences are noted in the text.


Post-MK-801
Pre-MK-801
Volume (VT)
Time (seconds)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.00
0.25
0.50
0.75
1.00
1.25
1.50

(from 133.6 ± 28.5 to 159.6 ± 32.5 br/min), V_T (from 1.18 ± 0.08 to 1.10 ± 0.08 ml), or V_E (from 159.3 ± 35.0 to 137.9 ± 42.1 ml/min) from levels observed during exposure to air, and there were no significant changes in the timing components of each breath (Fig. 6.5, 6.6). Hypercapnia, however, resulted in non-significant decrease in f_R (from 133.6 ± 28.5 to 129.3 ± 20.3 br/min) and a 156% increase in V_T (from 1.18 ± 0.08 to 3.03 ± 0.92 ml), resulting in a 145% increase in V_E (from 159.3 ± 35.0 to 389.0 ± 121.2 ml/min) (Fig. 6.5). Increases in V_T resulted from an increase in mean inspiratory flow rate (Fig. 6.6). Additional doses of MK-801 did not alter breathing further.

6.3.3. *Post - MK-801 treatment (CSF)*

Administration of MK-801 directly into the cerebrospinal fluid (CSF), rather than into the peripheral circulation also altered breathing. Subjectively, these results appeared to be identical to the results of intravenous injection. The low number of replicates precludes valid statistical assessment of these data and these have not been included in the analysis. Again, additional injections of MK-801 solution did not alter breathing further.

6.3.4. Hibernating animals

6.3.4.1. Control conditions

Resting ventilation in hibernating animals occurred in distinct multi-breath episodes separated by prolonged periods of apnea (Fig. 6.7). Before MK-801 treatment, there were no significant changes in breathing pattern associated with exposure to this level of hypoxia. Increasing the intensity of hypoxia, however, prompted these animals to arouse from hibernation. As such, it was not possible to characterize either the hypoxic ventilatory response

itself, or the effects of MK-801 on this response. Exposure to hypercapnia increased ventilation by 374 % (from 3.4 ± 0.5 to 16.1 ± 3.8 ml/min), primarily due to a 285 % increase in f_R (from 2.2 ± 0.4 to 8.6 ± 1.2 br/min) resulting from a 137 % increase in the number of breaths per episode (from 10.9 ± 1.3 to 25.8 ± 4.8) (Fig. 6.8, 6.9).

6.3.4.2. Post - MK-801 treatment

Injections of saline into the CSF had no influence on ventilation. Injection of MK-801 solution did not alter overall ventilation either but did result in an alteration of breathing pattern (Fig. 6.7). There was a 40 % increase in the number of breaths occurring in each breathing episode (from 10.9 ± 1.3 to 15.3 ± 2.5) and a decrease in T_{EP} of breaths within episodes resulting in a 25 % increase in the instantaneous frequency of breaths within breathing episodes (from 16.5 ± 1.3 to 20.6 ± 0.8 breaths per minute) (Fig. 6.8, 6.9). Subsequent to MK-801 treatment, there were still no significant changes in breathing pattern associated with exposure to hypoxia. Exposure to hypercapnia, however, increased ventilation by 370 % (from 3.3 ± 0.6 to 15.5 ± 4.6 ml/min), primarily due to a 330 % increase in f_R (from 2.0 ± 0.3 to 8.6 ± 1.5 br/min) resulting from a 108 % increase in the number of breaths per episode (from 15.3 ± 2.5 to 31.8 ± 6.4), and a 132 % increase in the frequency of breathing episodes (from 9.4 ± 1.4 to 21.8 ± 3.9 episodes per hour) (Fig. 6.8). Again, additional doses of MK-801 did not alter breathing further.

Fig. 6.7

Representative recordings of breathing from a hibernating squirrel before (Pre-MK-801) and after MK-801 treatment (Post-MK-801), during exposure to A) air, B) hypoxia (7% O_2) and C) hypercapnia (5% CO_2). Breathing records are from the integrated output of the pneumotachograph, with inspiration as an upward deflection and tidal volume proportional to the height of the deflection.

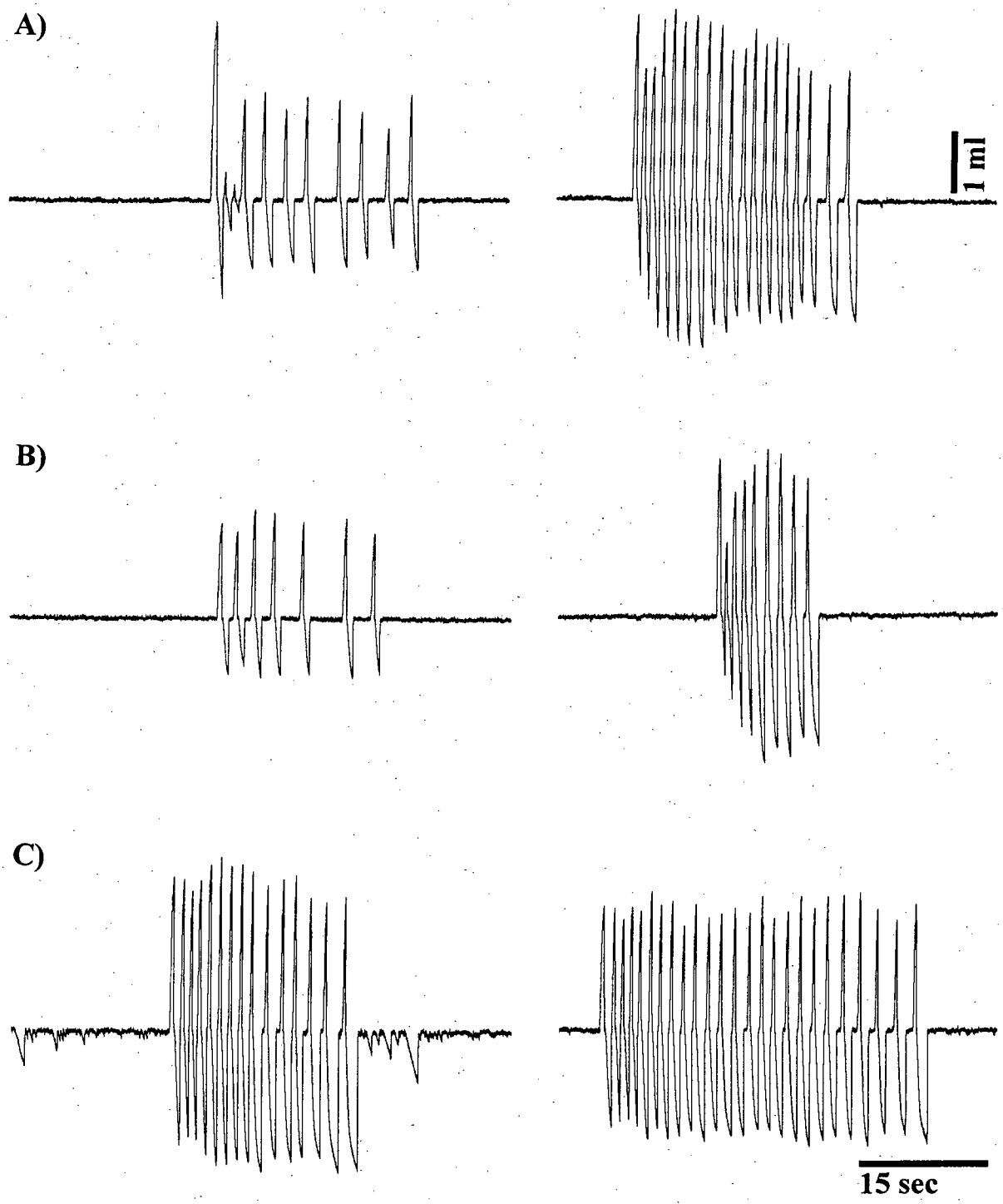

Pre MK-801
Post MK-801
A)
1 ml
B)
C)
15 sec

Fig. 6.8

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T), ventilation (V_E), episodes per hour, breaths per episode and the instantaneous frequency of breathing within episodes in hibernating squirrels during exposure to air, 10% O_2 and 5% CO_2 , before (open bars) and after (hatched bars) MK-801 treatment. The "*"denotes a significant difference from Pre-MK-801 values during exposure to air, while the "#" denotes a significant difference from Pre-MK-801 values during exposure to the same gas ($P<0.05$).

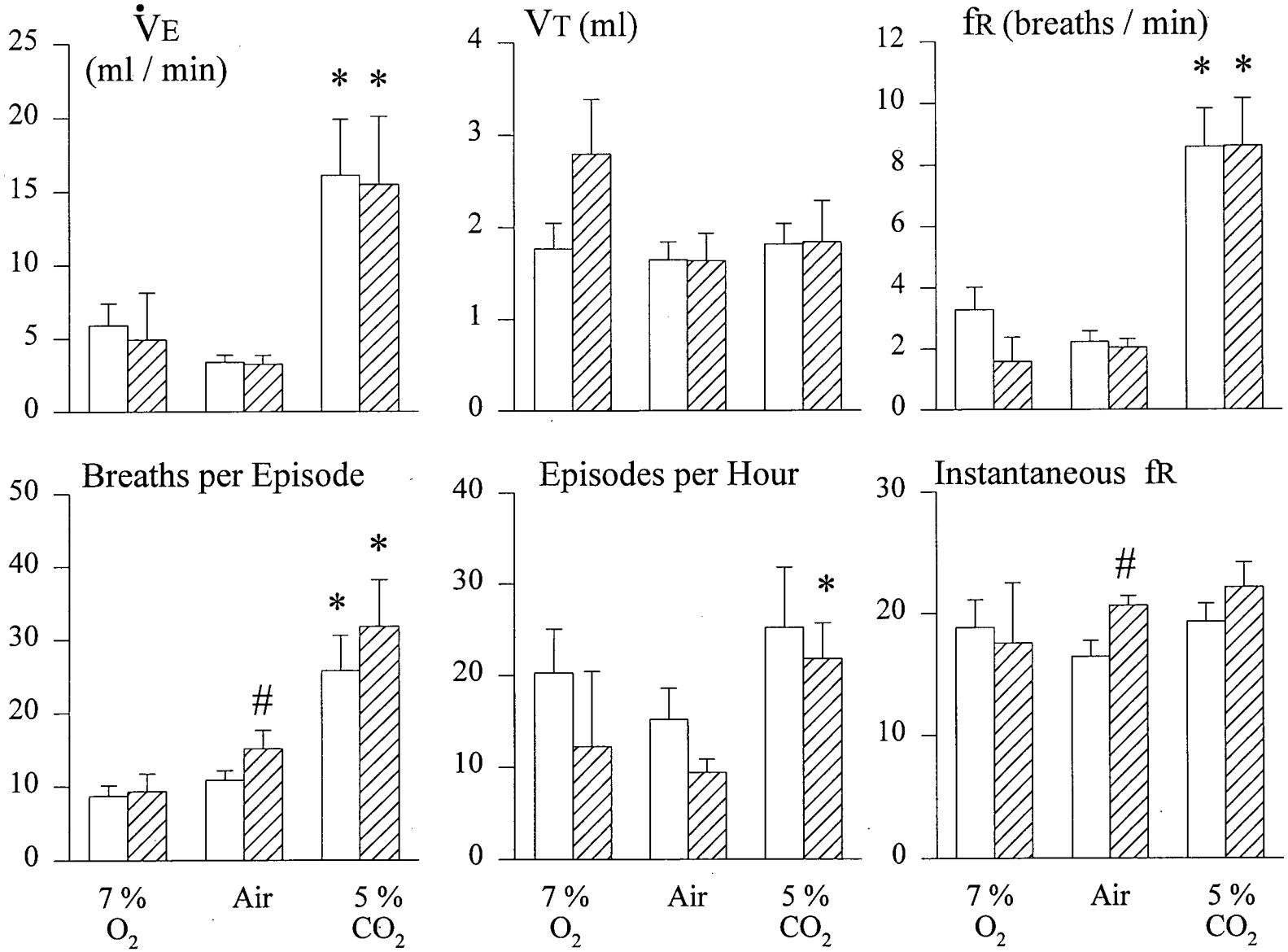
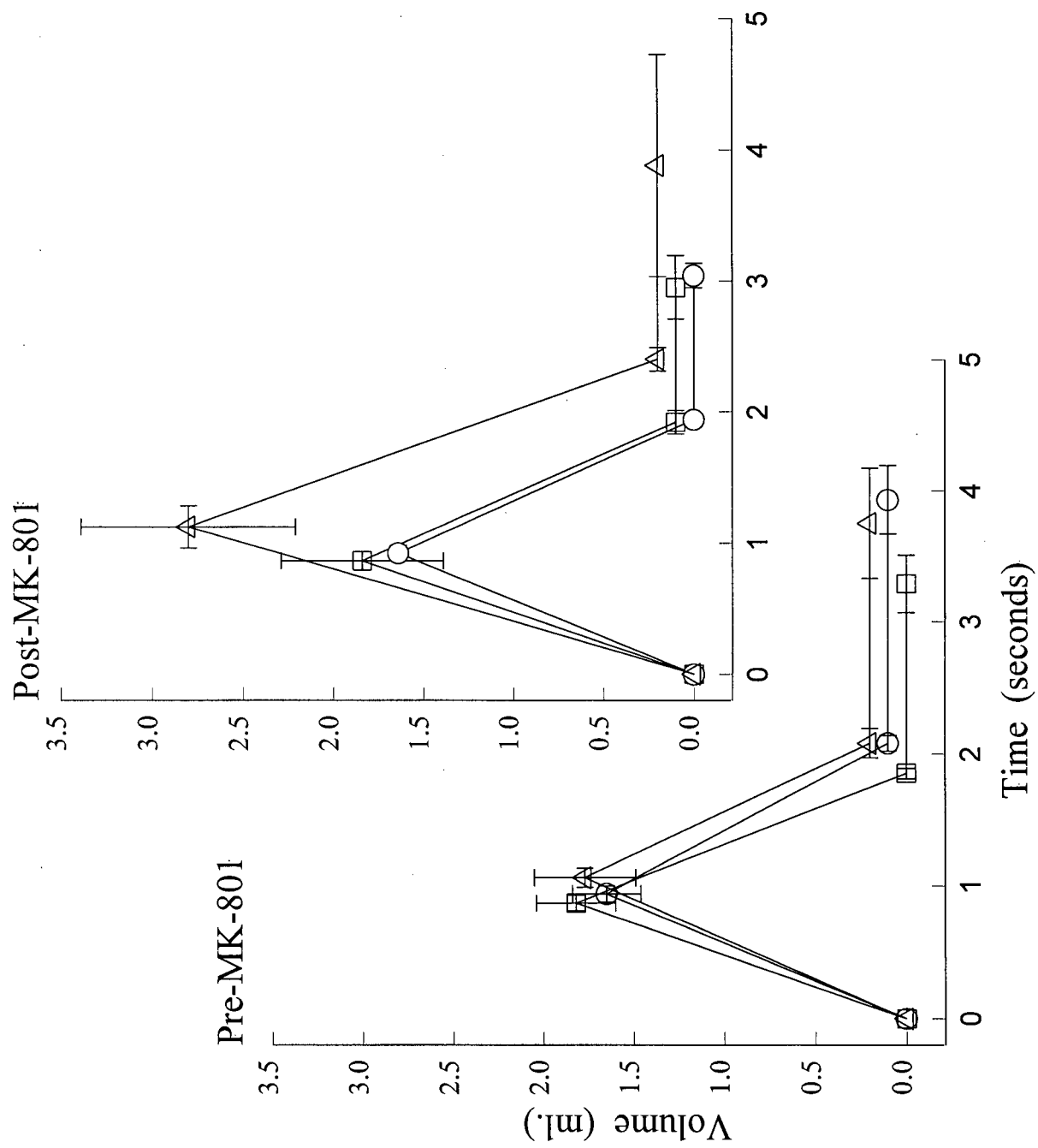

V̇E (ml / min)
VT (ml)
fR (breaths / min)
Breaths per Episode
Episodes per Hour
Instantaneous fR
7 % O2
Air
5 % CO2

Fig. 6.9

Representation of mean (± standard error) inspiratory volumes and durations of inspiration and expiration, including the end-expiratory pause in hibernating animals during exposure to air (○), 7% O_2 (△) and 5% CO_2 (□), before (Pre-MK-801) and after (Post-MK-801) MK-801 treatment. Statistical differences are noted in the text.


Post-MK-801
Pre-MK-801
Volume (ml.)
Time (seconds)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
1
2
3
4
5

6.4. Discussion

6.4.1. Euthermic animals

6.4.1.1. Initial ventilatory responses and influences of MK-801

Prior to MK-801 treatment, hypoxia produced non-significant increases in both V_T and f_R which resulted in significant increases in $\dot{V}_E$. This agrees with other observations in this (Webb and Milsom, 1994; Hunter et al. 1997) and other species (Hedmark and Kronenberg, 1982; Walker et al. 1985; Cragg and Drysdale, 1983).

Treatment with saline did not alter resting breathing and did not appear to have any significant influence on the hypoxic ventilatory response. Following MK-801 treatment, however, the trend for an increase in V_T, was eliminated and, thus, the increase in $\dot{V}_E$ was no longer significant. This is also consistent with observations from other studies (Ang et al. 1992; Mizuasawa et al. 1994; Miyawaki et al. 1996; Lin et al. 1995). In previous studies, antagonism of NMDA receptors within specific regions of the medulla, notably the NTS, abolished the increases in V_T which occurred in hypoxia, while the changes in f_R persisted. Input from carotid body chemoreceptors is transduced via the NTS, and these studies suggested that the stimulation of V_T by hypoxia required NMDA receptor-mediated processes at this site. The trends in the data in the present study are consistent with this hypothesis but the modest responses and lack of significant changes in f_R or V_T make further speculation pointless.

Prior to MK-801 treatment, hypercapnia produced significant increases in V_T but decreases in f_R, the net result being significant increases in $\dot{V}_E$. Depression of f_R is not a common feature of the hypercapnic ventilatory response in other studies of this species (Webb and Milsom, 1994; Hunter et al. 1997) or studies in other species (see Cunningham et al. 1986 for review). While MK-801 treatment did not significantly change ventilation during exposure

to air or hypoxia it significantly increased fR and decreased VT during hypercapnia. These influences were offsetting and there was no difference between the level of VE observed during hypercapnic exposure before and after MK-801 treatment. The hypercapnic ventilatory response after MK-801 treatment now consisted of only a modest increase in VT.

The ameliorated increases in VT associated with hypercapnia following MK-801 and that associated with hypoxia can not be due to the same mechanism. While Mizusawa et al. (1994) showed that antagonism of NMDA receptors within the NTS prevented the increases in VT associated with exposure to hypoxia, it did not reduce the changes in VT which occurred during the hypercapnic ventilatory response. NMDA antagonism in the ventrolateral medulla, however, does remove the increases in the amplitude of phrenic activity associated with the hypercapnic response, suggesting that the reductions in VT noted in the present investigation may be mediated by NMDA receptor-mediated processes in this area (Dillon et al. 1991; Nattie and Li, 1995; Nattie et al. 1993).

The fall in fR during hypercapnia was eliminated following MK-801 treatment suggesting that this decline was due to an NMDA receptor-mediated process. Ablation of the PBC has been shown to remove the fR component of the hypercapnic ventilatory response of anesthetized or decerebrate cats (St. John, 1977). This response was an increases in fR, however, and not a decrease as in the present study. It is not known whether the factors within the PBC responsible for this change were influenced through NMDA or non-NMDA receptor-mediated processes. Thus, it is difficult to speculate where the results noted in the present study originate.

6.4.2. Anesthetized animals

6.4.2.1. Initial ventilatory responses and influences of MK-801

Prior to MK-801 treatment, hypoxia produced no significant change in V_T, but did produce significant increases in f_R and V_E. This agrees with other observations in this species during anesthesia (Hunter et al. 1997; chapter 4). Anesthesia alone depresses f_R and V_T. Following anesthesia, hypoxia produces greater increase in f_R and has no influence on V_T.

Treatment with MK-801 stimulated breathing in anesthetized animals. Following MK-801 treatment, f_R and V_E during exposure to air were significantly elevated. These data indicate that the depression of f_R associated with anesthesia may be due to NMDA receptor-mediated processes (chapter 5). Following MK-801 treatment, hypoxia still did not produce an increase in V_T, but it also no longer stimulated f_R. The hypoxic ventilatory response was, therefore, abolished by MK-801 treatment. More specifically, ventilation while breathing air following MK-801 was elevated to levels equivalent to those observed during hypoxia before MK-801 treatment, and failed to increase further with subsequent hypoxia.

Both anesthetized dogs and unanesthetized rats also exhibit a reduced hypoxic ventilatory response following MK-801 treatment (Ang et al. 1992; Mizusawa et al. 1994). In these studies, as in the unanesthetized squirrels, it was the increase in V_T in response to hypoxia which was abolished by MK-801. The increase in f_R still remained. In the anesthetized squirrels, however, changes in V_T were already eliminated by the anesthesia and now, following MK-801, the animals fail to exhibit further increases in f_R in response to hypoxia. This is quite different than the influence of MK-801 seen in other species. The lack of an increase in f_R during hypoxia following MK-801 suggests that either such an alteration requires NMDA receptor-mediated processes, or that f_R has risen to a maximum level due to the actions

of MK-801 treatment alone. The levels of f_R achieved following MK-801 treatment were equal to those observed in unanesthetized animals during exposure to either hypoxia or hypercapnia, and were as high as the rate produced by hypercapnia under any condition. In addition, the increase in f_R in animals exposed to air following MK-801 treatment suggest that MK-801 would not prevent an increase in f_R during hypoxia.

Prior to MK-801 treatment, hypercapnia produced significant increases in V_T and non-significant increases in f_R, resulting in significant increases in V_E. This response, too, is similar to previous observations of anesthetized ground squirrels (Hunter et al. 1997). Anesthesia alone creates an overall depression of f_R and V_T. Following anesthesia hypercapnia produced a non-significant increase in f_R rather than a significant decrease and a smaller increase in V_T. These changes are similar to the influences of anesthesia on the hypoxic ventilatory response.

MK-801 treatment had no significant influence on the overall hypercapnic ventilatory response although the increases in f_R previously observed during this response were eliminated. St.John (1977) demonstrated that lesions within the PBC eliminated the increase in f_R exhibited by decerebrate cats during hypercapnia, while the increase seen in V_T remained. Thus, a loss of NMDA receptor function within the PBC could account for the absence of any increase in f_R during hypercapnia in the present study. Alternatively, as discussed with the hypoxic ventilatory response, f_R may have been accelerated to a maximum level by MK-801 treatment alone.

The increases seen in V_T during hypercapnia in unanesthetized animals, prior to MK-801 was much greater than it was in anesthetized animals. The increase in V_T during hypercapnia in unanesthetized animals after MK-801 was similar to that in anesthetized

animals. This suggests that anesthesia reduces V_T by inhibiting an NMDA receptor-mediated process. Since this process is already inhibited by anesthesia, the increase seen in V_T with hypercapnia was not as great and was not altered by MK-801 treatment in anesthetized animals whereas the much greater increase seen in unanesthetized animals was greatly reduced by MK-801.

6.4.3. Hibernating animals

6.4.3.1. Initial ventilatory responses

Prior to MK-801 treatment, hibernating squirrels exhibited an extremely robust ventilatory response to hypercapnia, when compared to that of euthermic animals, consistent with previous investigations (McArthur and Milsom, 1991; Harris and Milsom, 1994). The hypercapnic response was mediated by an increase in f_R, resulting from an increase in the number of breaths occurring in each breathing episode. Also consistent with a previous study, animals in the present investigation did not exhibit a significant ventilatory response to 7 % O_2 (McArthur and Milsom, 1991b). These authors note, however, that this species does increase ventilation when exposed to more extreme levels of hypoxia, ranging from 5 and 3 % O_2. When animals in the present investigation were exposed to less than approximately 6 % O_2 they failed to maintain the hibernating state and initiated arousal from hibernation. As such, the hypoxic ventilatory response could not be characterized during this investigation.

6.4.3.2 Post MK-801 treatment

As was demonstrated in chapter 5, antagonism of NMDA receptors resulted in an increase in the instantaneous breathing frequency within each breathing episode, and in the

number of breaths occurring in these episodes. Hypoxia subsequent to MK-801 treatment still did not significantly alter breathing. Hypercapnia, however, resulted in an increase in f_R, mediated by increases in both the number of breaths occurring in each breathing episode and the frequency of breathing episodes themselves. These results indicate, therefore, that although resting ventilation may be influenced by NMDA antagonism, these receptors are not involved in the hypercapnic ventilatory response during hibernation.

6.5. Conclusion

Hypoxic responses in unanesthetized animals involve increases in tidal volume and breathing frequency. NMDA receptor-mediated processes appear to contribute to the increase in V_T. Anesthesia eliminates the hypoxic influence on V_T. Now the hypoxic ventilatory response involves only an increase in f_R. Anesthesia depresses f_R by an NMDA receptor-mediated process. Hypoxia can reverse this in part. This level of hypoxia had no significant influence in hibernation.

The hypercapnic ventilatory response involves decreases in f_R and increases in V_T. NMDA receptor-mediated processes contribute to the increases in V_T but via a different mechanism than hypoxia. These same processes may have contributed to the decrease in f_R. In anesthesia, the hypercapnic ventilatory response now involves an increase in both f_R and V_T. NMDA receptor-mediated processes contribute to the increase in f_R only. The NMDA receptor-mediated increase in V_T seen in unanesthetized animals appears to be anesthesia-sensitive, as does the decrease in f_R. The hypercapnic ventilatory response of hibernating squirrels was unaffected by MK-801.

Chapter 7

The Influence of Interactions Between Vagal Feedback and NMDA Receptor-Mediated Processes on Breathing Pattern and Ventilatory Chemoresponses in Ground Squirrels

Abstract

The influences of blockade of the function of N-methyl-D-aspartate (NMDA) type glutamate receptors by the non-competitive antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801), in conjunction with the removal of vagal afferent feedback, on breathing pattern and ventilatory chemoresponses, were assessed in the golden-mantled ground squirrel, *Spermophilis lateralis,* during anesthesia and natural hibernation.

Systemic injection of MK-801 in anesthetized vagotomized squirrels induced apneusis. Despite the prolongation of inspiratory duration V_T was not increased significantly during apneusis. VT was increased by hypercapnia, however, indicating that inspiration was not maximal during apneusis but could be increased by stimulation of ventilatory drive. Hibernating animals exhibited an episodic breathing pattern. Injection of MK-801 into the cerebrospinal fluid of hibernating squirrels in conjunction with vagal blockade converted the episodic breathing pattern into one of more evenly spaced single breaths and eventually induced arousal from hibernation. It did not result in apneusis.

These data indicate that in euthermic animals vagal feedback and NMDA receptor-mediated processes interact to terminate inspiration and produce normal ventilatory burst pattern formation. In hibernating animals, these same inputs (vagal feedback and NMDA receptor-mediated processes) appear to be involved in clustering breaths into episodes and play little or no role in terminating individual breaths.

7.1. Introduction

In chapter 3, it was demonstrated that ventilation in euthermic golden-mantled ground squirrels was critically dependant on afferent information transmitted through intact vagus nerves. These squirrels did not exhibit spontaneous ventilation in the absence of vagal feedback. In anesthetized animals, on the other hand, ventilation continued post-vagotomy, but the shape of individual breaths was altered. Breathing was slower and deeper indicating that while vagal feedback was no longer critical, it still played an important role in determining breathing pattern. In hibernating animals, vagal feedback was even less critical, it increased the overall level of ventilation by increasing the length of breathing episodes, but did not influence the frequency or depth of individual breaths within each episode.

In chapter 5 it was demonstrated that ventilation in euthermic golden-mantled ground squirrels was not influenced by blockade of the function of N-methyl-D-aspartate (NMDA) type glutamate receptors by the non-competitive antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801), while in anesthetized animals, ventilation was stimulated by MK-801 treatment suggesting that glutamate, acting by NMDA receptors, inhibited breathing during anesthesia. In hibernating animals MK-801 treatment elevated both the number of breaths in each breathing episode, and the frequency with which these breaths occurred.

The data from these previous studies indicate, thus, that vagal feedback facilitates breathing, more in unanesthetized animals than anesthetized or hibernating animals, whereas glutamatergic NMDA receptor-mediated processes, interrupted by MK-801, depress breathing in anesthetized and hibernating animals but not in unanesthetized animals. Questions now arise concerning the interaction of vagal feedback with NMDA receptor-mediated processes; what

will be the influence of MK-801 treatment combined with vagotomy ?

Previous studies have demonstrated that the antagonism of NMDA-type glutamate receptors with systemic application of MK-801 mimics the influences of specific lesions within the dorsal pons (comprising the medial and lateral Parabrachial and Kölliker-Fuse nuclei, and referred to as either the pneumotaxic center, pontine respiratory group, or Parabrachial complex (PBC)) on breathing pattern in cats and rats (Ling et al. 1994, Fung et al. 1994). Transections which remove this area, or electrolytic or pharmacological lesions within this region, made in conjunction with removal of afferent vagal inputs results in a profound disruption of breathing. The resulting breathing pattern, consisting of abnormally prolonged inspirations interrupted by short expirations, is referred to as apneusis. This disruption has been characterized most closely in cats but has been observed in many other mammalian species (Foutz et al. 1988; Connelly et al. 1992; Wang et al. 1993; Ling et al. 1994; Fung et al. 1994; St.John 1979; Cassus-Soulanis et al. 1995).

Wang et al. (1993) have suggested that the rodent respiratory control network is identical to that of the cat. Interestingly, however, not all investigations have been able to produce apneusis in rodents by vagotomy and disruption of the PBC (Montreau et al. 1989; Montreau et al. 1990; Connelly et al. 1992; Jodkowski et al. 1994, Cassus-Soulanis et al. 1995). While some investigators have used these data to question the relative importance of interactions between vagal feedback from pulmonary receptors and pontine influences in the control of breathing pattern in general, others have suggested that there could be confounding influences of specific anesthetics, or species and strain-specific differences between rodent types which might explain the absence of apneusis in some vagotomized rodents treated with NMDA-type glutamatergic antagonists (Connely et al. 1992, Cassus-Soulanis et al. 1995).

Given this background it is very difficult to predict what the influences of combined vagotomy and MK-801 treatment would be in squirrels. Vagotomy has a far more profound influence on breathing in unanesthetized squirrels (eliminating ventilation) than has been shown in other species. MK-801 treatment has no effect in these animals, whereas it depresses ventilation in most other species. Finally, whereas vagotomy and MK-801 treatment typically produce apneusis in most mammals, they do not consistently have this result in rodents.

In light of this, the present study was designed to examine the breathing pattern of unanesthetized, anesthetized and hibernating squirrels, and their ventilatory responses to hypoxia and hypercapnia, before and after antagonism of NMDA receptors combined with vagotomy or vagal blockade. The fundamental question being addressed was how do vagal feedback and NMDA receptor-mediated processes, within the PBC and elsewhere, interact to influence breathing pattern.

7.2. Methods

7.2.1. Instrumentation

Experiments were performed on the same ground squirrels *(Spermophilus lateralis)* described in previous chapters. Animals used in the chronic studies were surgically instrumented *a priori*. Animals were fitted with four electroencephalographic (EEG), two electrocardiographic (ECG), two electromyographic (EMG) and two respiratory impedance electrodes, a third cerebral ventricle cannula and a vagal infusion cuff following contralateral vagotomy as outlined in the general methods section (chapter 2).

7.2.2 Protocol

7.2.2.1. Euthermic animals (chronic study)

Six squirrels selected for this portion of the study underwent surgery during the summer. Unanesthetized animals were transferred to a whole body plethysmograph. The cannula supplying the vagal cuff was connected to a 3 cc. syringe via a length of polyethylene tubing (PE 50). The syringe and tubing were filled with xylocaine except for the final 0.2 ml at the tip which was filled with saline, separated from the xylocaine by a small air bubble. The "dummy" cannula was replaced with a 23-gauge "injection" cannula (Plastics One , Roanoke VA.) which was connected to a 10 µl Hamilton syringe via a length of polyethylene tubing (PE 20). The syringe and tubing were filled with a solution of the non-competitive NMDA-type glutamatergic antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801, Research Biochemicals) in saline (5 mg/ml, 15 mM; pH 7.3 ±0.1) except for the final 2 µl at the tip which was filled with saline, separated from the MK-801 solution by a small (<0.2 µl) air bubble.

Animals were supplied with air and monitored for at least 2 hours. They were then randomly exposed to hypercapnic (5% CO_2 in air) and hypoxic (10% O_2 in nitrogen) gas mixtures for at least 30 minute periods separated by 30 minutes of exposure to air. Animals were then monitored for 30 minutes while exposed to air following a 2 µl injection of saline (sham) into the third ventricle, and 30 minutes following a 2 µl injection of MK-801 solution. Animals were again randomly exposed to hypercapnic and hypoxic gas mixtures separated by 30 minutes of exposure to air. Additional injections of MK-801 solution were repeated every 30 to 45 minutes. Animals were then monitored for one hour following a 0.2 ml infusion of saline (sham) into the vagal cuff, and again following infusion of 2% xylocaine (vagal blockade) into the vagal cuff.

7.2.2.2. Hibernating animals (chronic study)

Seven animals selected for this portion of the study underwent surgery during the winter. Following recovery, these animals reentered hibernation. During deep hibernation, animals were transferred to a plexiglass box (12 x 20 x 10 cm) within a laboratory controlled-environment chamber. Each box was supplied with air at a flow rate of 500 ml per minute. Ambient temperature was held at 5 °C. Animals were fitted with a mask connected to a resistance pneumotachograph to measure ventilation. The cannula supplying the vagal cuff was connected to length of xylocaine-filled polyethylene tubing and a syringe, and the "dummy" cannula was replaced with an "injection" cannula connected to a 10 µl Hamilton syringe as described above.

Animals were monitored; i) for at least two hours to provide initial control values, ii) for at least two hours while the test box was supplied with hypercapnic (5% CO_2 in air) gas, iii)

for at least two hours while the test box was supplied with hypoxic (7% O_2 in N_2) gas, iv) for at least one hour during exposure to air following a 2 µl injection of saline (sham treatment) into the third ventricle and, v) for at least one hour following a 2 µl injection of MK-801 solution. Supplemental (2 µl) injections of MK-801 solution were made every hour and animals were monitored, during exposure to air, following a 0.2 ml infusion of saline (sham treatment) into the vagal cuff and again following a 0.2 to 0.4 ml injection of xylocaine (vagal blockade) into the vagal cuff.

7.2.2.3. Additional trials

In three animals, trials were repeated but vagal blockade was induced during exposure to 5% CO_2 subsequent to MK-801 treatment to determine the results of combined treatment during periods of elevated ventilatory drive. In four animals, trials were repeated and the procedures for MK-801 treatment and vagal blockade were reversed such that the influences of MK-801 treatment were assessed subsequent to vagal blockade.

7.2.2.4. Anesthetized animals (acute study)

Seven ground squirrels were used in this series of experiments. Animals were anesthetized, tracheotomized, fitted with EEG, ECG, EMG and respiratory impedance electrodes. Their left and right cervical vagi were isolated and snared with suture, and catheters were implanted in a femoral artery and vein, as described in Chapter 2.

Animals were supplied with air and monitored for at least 2 hours, and then randomly exposed to hypercapnic (5% CO_2 in air) and hypoxic (10% O_2 in nitrogen) gas mixtures for at least 30 minute periods separated by 30 minutes of exposure to air. Following hypoxic and

hypercapnic treatment, animals were returned to air and monitored for at least one hour following a 0.3 ml intravenous infusion of saline (sham) into the venous catheter, and again following a 0.2 to 0.3 ml infusion of a 0.15 mg/ml (0.45 mM) solution of MK-801 in saline (pH 7.3 ±0.1) resulting in a dose of 0.15 - 0.30 mg MK-801 per kg body mass.

At least one hour later animals were returned to air and underwent bilateral cervical vagotomy. Vagotomy was achieved by injecting 0.2 ml of xylocaine into the previously implanted polyethylene sleeves, and then severing the nerves by pulling the loop of suture through each sleeve. At least 30 minutes after vagotomy, animals were again exposed to hypoxic and hypercapnic gas mixtures. Additional 0.2 to 0.3 ml infusions of MK-801 solution were administered every hour.

7.2.2.5. Additional trials

In three additional squirrels, trials were repeated but the procedures for MK-801 treatment and vagotomy were reversed such that the influences of MK-801 treatment were assessed subsequent to vagotomy. Hypoxic and hypercapnic ventilatory responses were not assessed during these trials.

7.3. Results

7.3.1 Euthermic animals

7.3.1.1. Initial values

Before MK-801 treatment, hypoxia produced non-significant increases in both respiratory frequency (f_R) and tidal volume (V_T) which together resulted in a significant increase in ventilation (V_E) without significantly altering the timing components of each breath,

while hypercapnia decreased f_R and increased V_T, resulting in an increase in $\dot{V}_E$. The decrease in f_R stemmed from an increase in the durations of the end-expiratory pauses (T_{EP}) and the duration of inspiration (T_I) (chapter 6).

7.3.1.2. Post - MK-801 treatment and vagal blockade

Breathing was not significantly altered by either intravenous injections of saline or MK-801 treatment. Subsequent sham injections of saline had no effect on breathing. Vagal blockade subsequent to MK-801 treatment, however, resulted in an almost immediate cessation of breathing. Breathing occurred only in "gasp-like" breaths separated by prolonged periods of apnea. Animals were immediately removed from the plethysmograph and artificially ventilated in an attempt to restore spontaneous ventilation. Such attempts were unsuccessful and spontaneous ventilation was never restored. Recordings of respiratory impedance suggested apneas were central and not obstructive, as there were no indications of body wall movement and attempted breathing.

7.3.2 Anesthetized animals

7.3.2.1. Initial values

Before MK-801 treatment, hypoxia produced an increase in f_R, stemming from a decrease in T_{EP}, and a non-significant decrease in V_T, and in an increase in $\dot{V}_E$. Hypercapnia produced an increase in f_R, stemming from a decrease in T_{EP}, an increase in V_T, stemming from an increase in mean inspiratory flow rate, and an increase in $\dot{V}_E$ (Fig. 7.1, 2, 3).

Fig. 7.1

Representative recordings of breathing in an anesthetized squirrel exposed to A) air, B) hypoxia (10% O_2) and C) hypercapnia (5% CO_2) Before (Pre-treatment) and after MK-801 treatment and vagotomy (MK-801 + VX). Breathing records in the upper tracings are the integrated output of the pneumotachograph, with inspiration as an upward deflection and tidal volume proportional to the height of the deflection. The lower tracings are impedance measurements representing the inflation state of the chest. Note that before treatment, inspiration is associated with chest inflation. The chest deflates on expiration and remains at a neutral state during an end-expiratory pause (TEP). In contrast, during the apneustic breaths associated with MK-801 + VX the chest inflates on inspiration and the inflation is maintained during an end-inspiratory pause (TIP).

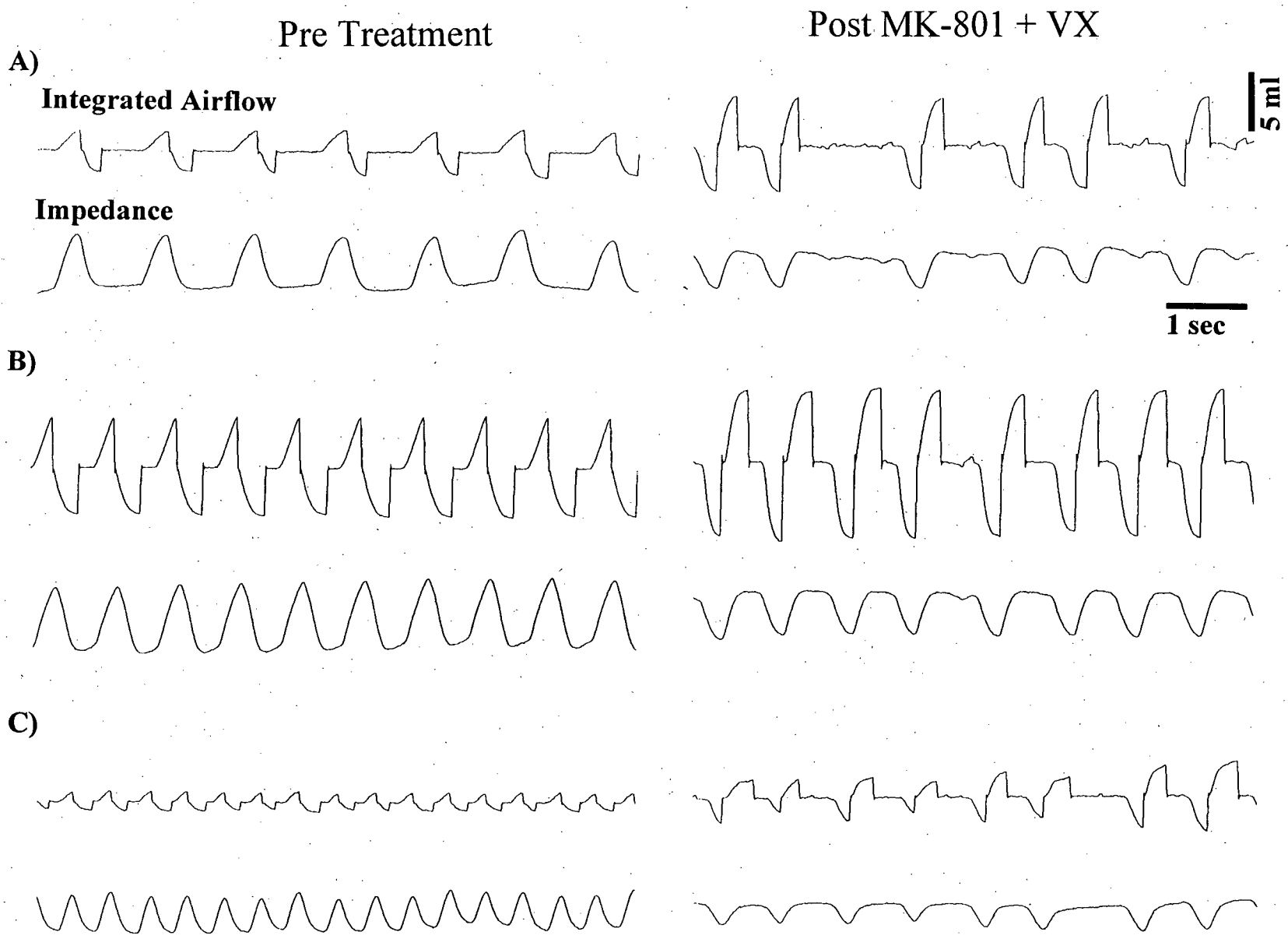

Pre Treatment
Post MK-801 + VX
A)
Integrated Airflow
Impedance
5 ml
1 sec
B)
C)

Fig. 7.2

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) during exposure to air, 10% O_2 and 5% CO_2 of anesthetized squirrels before treatment (open bars) and following MK-801 treatment and vagotomy (hatched bars). The "*" denotes a significant difference from the value during exposure to air in that treatment, while the "#" denotes a significant difference from pre-treatment values during exposure to that gas ($P<0.05$).

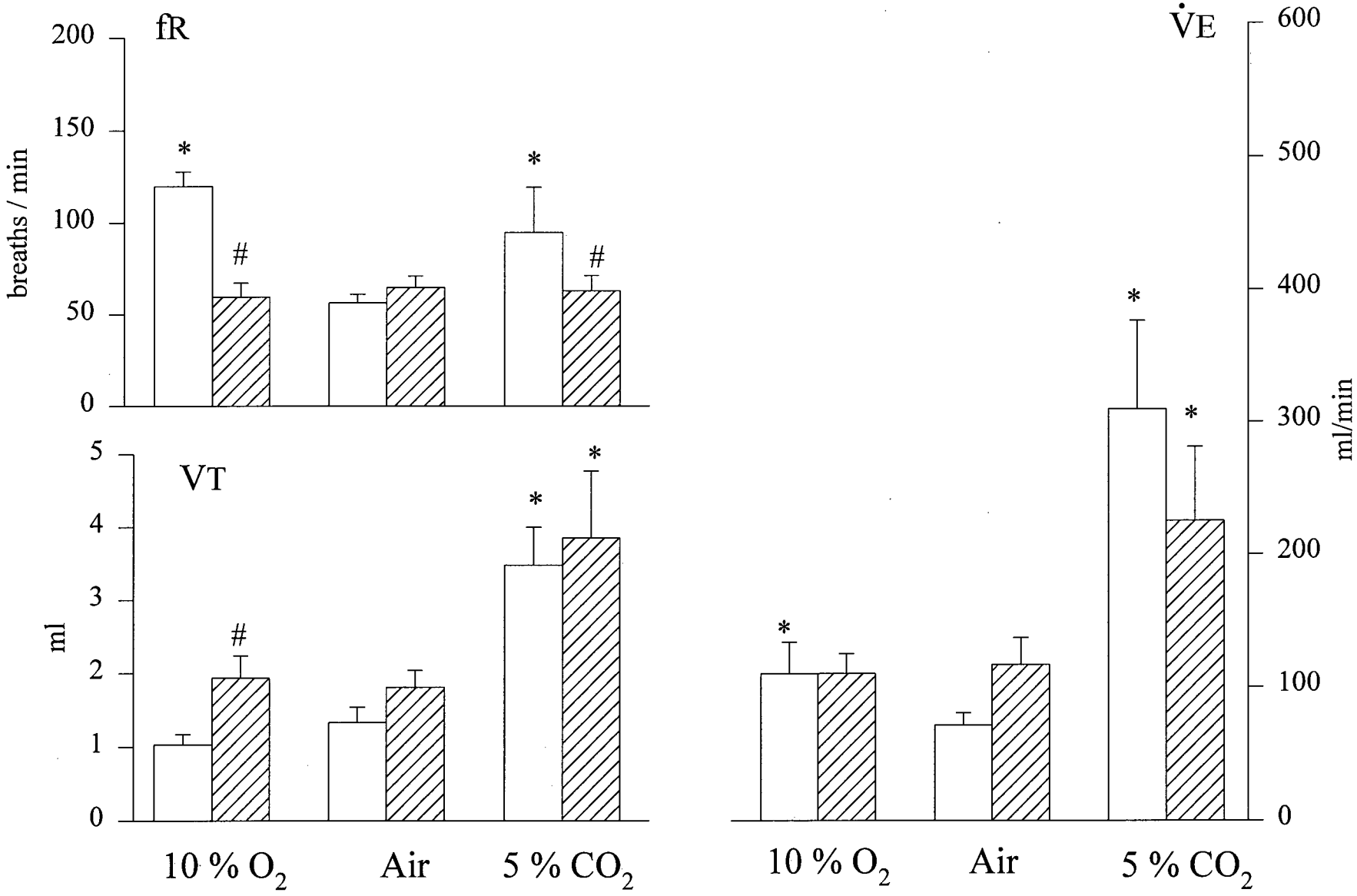
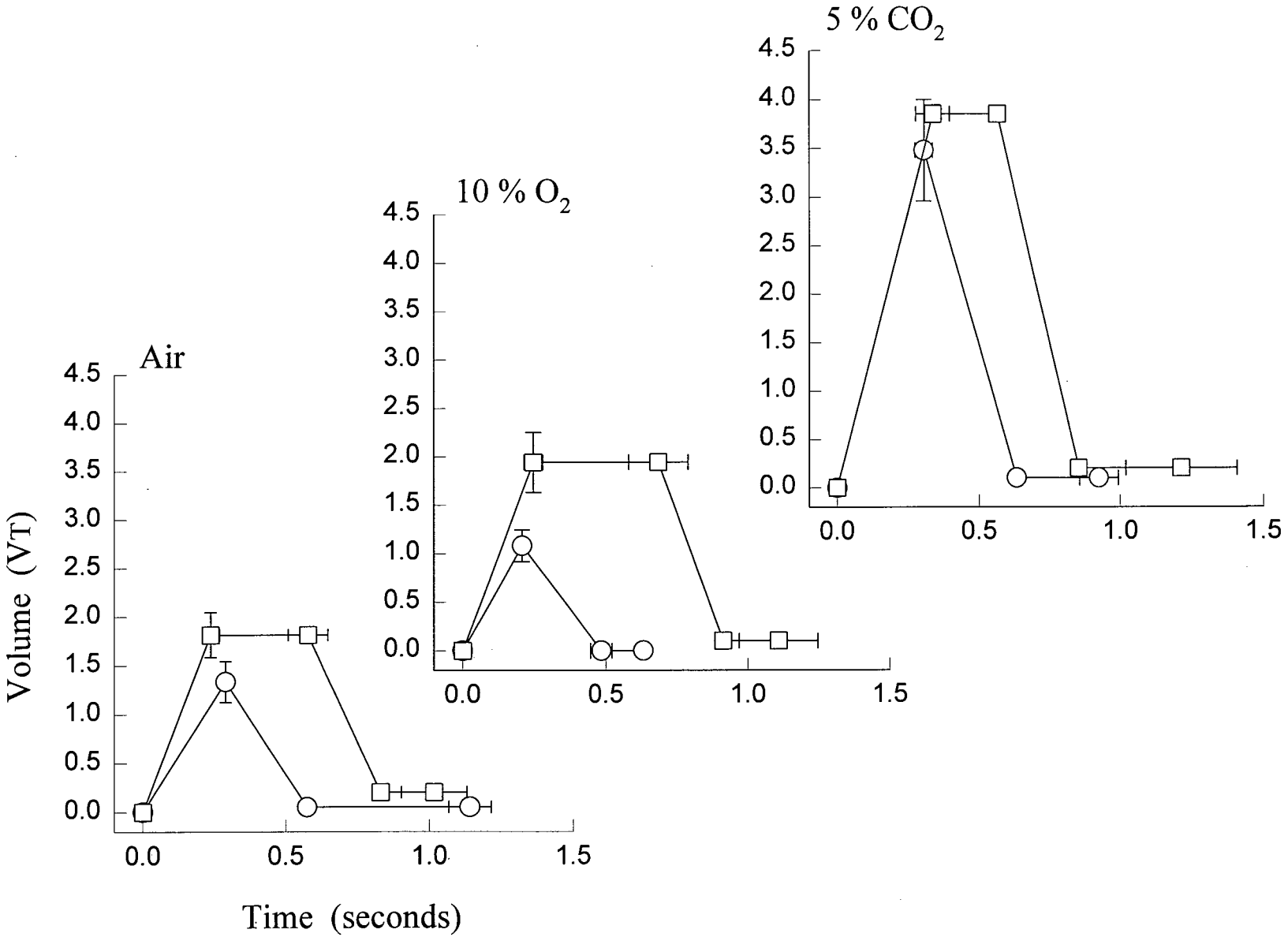

fR
breaths / min
200
150
100
50
0
VT
ml
5
4
3
2
1
0
$\dot{V}E$
ml/min
600
500
400
300
200
100
0
10 % O_2
Air
5 % CO_2
10 % O_2
Air
5 % CO_2

Fig. 7.3

Representation of mean (± standard error) inspiratory volumes and durations of inspiration and expiration in anesthetized animals before treatment (○) and following MK-801 treatment and vagotomy (□), during exposure to air, 10% O_2 and 5% CO_2. Statistical differences are noted in the text. Note that in different plots the end-expiratory volumes have been offset from zero for clarity.


Air
10 % O2
5 % CO2
Volume (VT)
Time (seconds)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

7.3.2.2. Post - MK-801 treatment and vagotomy

Intravenous injections of saline had no influence on breathing. Following MK-801 treatment, however, there was an increase in f_R, stemming from a decrease in T_{EP} and resulting in an increase in $\dot{V}_E$. Vagotomy subsequent to MK-801 treatment did not alter ventilation from that observed during eupnea before MK-801 treatment. (Fig. 7.2). The timing components of the individual breaths, however, were altered (Fig. 7.3). Most notable was the increase in the duration of inspiration (T_I). Mean inspiratory flow rate and the duration of inspiratory flow (T_{IA}) were unchanged. The prolongation of inspiration was due to the occurrence of a end-inspiratory pause (T_{IP}) which preceded expiration. This characteristic end-inspiratory pause reflected the development of an apneustic pattern of breathing. There was no change in the duration of the ventilatory cycle (T_{TOT}) as the duration of expiration (T_E) decreased, predicated by a decrease in the duration of the end-expiratory pause (T_{EP}), while the duration of expiratory airflow (T_{EA}) was unchanged.

Following vagotomy and MK-801 treatments, hypercapnic exposure resulted in an increase in V_T, T_{IA}, $\dot{V}_E$ and mean inspiratory flow rate, although, f_R, T_{EA}, T_{EP} and T_{IP} were all not significantly different than during air exposure. None of the ventilatory variables were changed by hypoxia (Fig. 7.2,3). Supplemental doses of MK-801, did not alter the apneustic breathing pattern further.

7.3.2.3. Additional trials

In three additional squirrels, trials were repeated but the procedures for MK-801 treatment and vagotomy were reversed such that the influences of MK-801 treatment were assessed subsequent to vagotomy. Vagotomy itself altered ventilation by decreasing f_R and

increasing V_T. Ventilation was stimulated by both hypoxia and hypercapnia following vagotomy, although ventilatory responses were dampened (chapter 4). Subsequent MK-801 treatment resulted in apneustic breathing which was identical to that outlined in the previous section.

7.3.3 Hibernating animals

7.3.3.1. Initial values

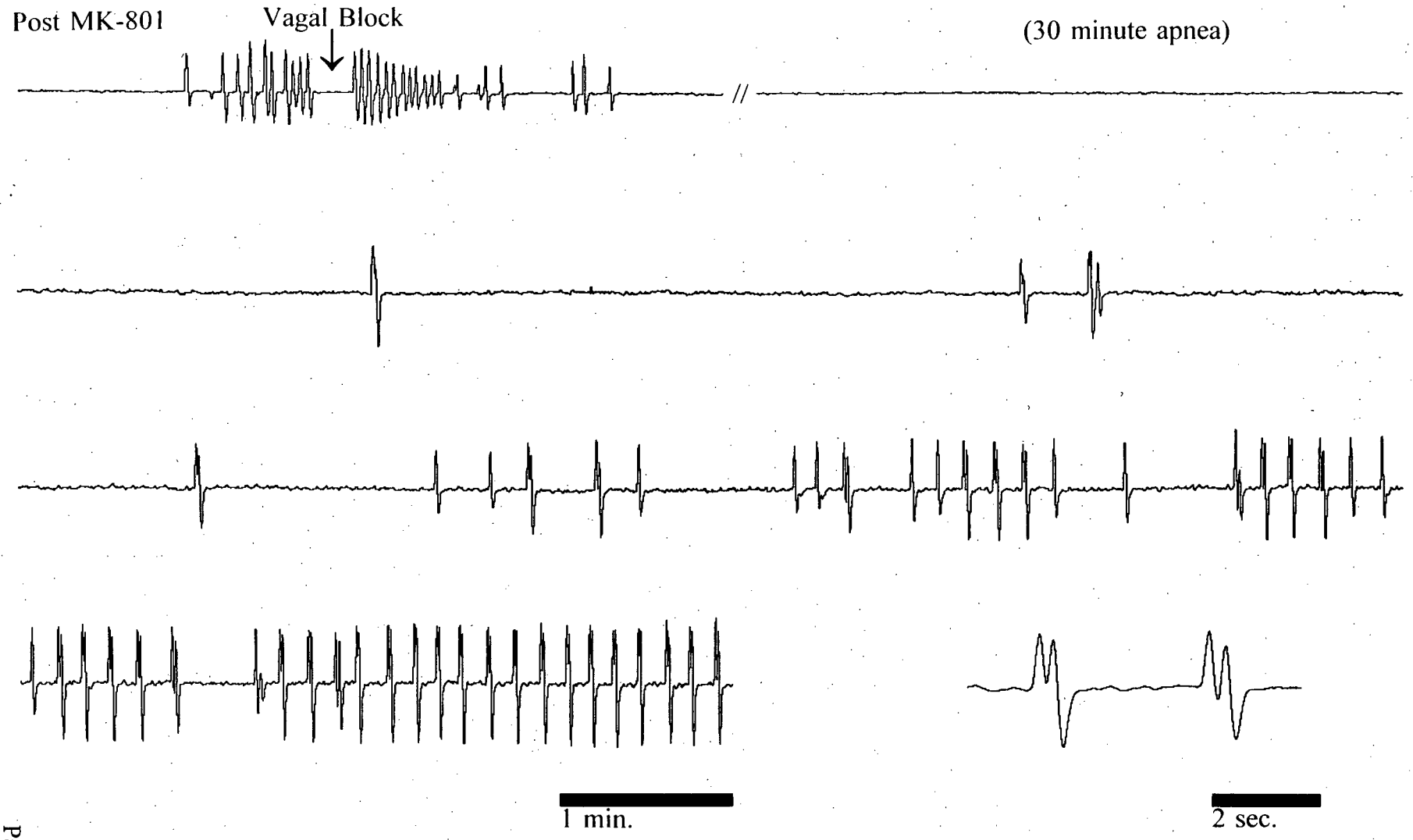
Resting ventilation in hibernating animals occurred in distinct multi-breath episodes separated by prolonged periods of apnea. Before MK-801 treatment, there were no significant changes in breathing pattern associated with exposure to hypoxia, while hypercapnia increased ventilation by increasing f_R through an increase in the number of breaths per episode (chapter 4).

7.3.3.2. Post - MK-801 treatment and vagal blockade

Injections of saline into the CSF had no effect. Injection of MK-801 solution did not alter overall ventilation but did result in an alteration of breathing pattern, resulting in an increase in the number of breaths occurring in each breathing episode and a decrease in T_{EP} of breaths within episodes. This resulted in an increase in the instantaneous frequency of breaths within breathing episodes. Following vagal blockade subsequent to MK-801 treatment, breathing episodes no longer occurred. Instead, the period of apnea following the final breathing episode was now punctuated by a series of single breaths (Fig. 7.4). The shapes of the individual breath were altered from those that typically occurred before this treatment. The duration of the inspiratory phase of the respiratory cycle was increased and, subjectively, it

Fig. 7.4

Representative recordings of breathing in a hibernating squirrel illustrating the influence of vagal blockade administered subsequent to MK-801 treatment. There is a 25 minute break in the upper record during which apnea occurred but the remainder of this trace is continuous with the lower three traces. An expanded record of a single breath (inset lower right) illustrates the "twinned" inspiratory peaks. The breathing record is the direct output of the pneumotachograph, with inspiration as an upward deflection and tidal volume proportional to the area under the deflection.


Post MK-801
Vagal Block
(30 minute apnea)
1 min.
2 sec.

appeared that the inspiratory actions of intercostal and diaphragm muscles were often uncoupled. Whatever the cause, breaths now commonly occurred as "twinned" inspirations followed by a single expiration (Fig. 7.4). Invariably, there was a gradual increase in the frequency of single breaths, and arousal from hibernation occurred. The arousal from hibernation precluded the characterization of ventilatory responses to either hypoxia or hypercapnia following combined treatment. Once arousal from hibernation was initiated, observations were discontinued. The animal's vagal cuff was flushed with saline and they were left to arouse at room temperature. At no time was apneusis observed during arousal from hibernation.

7.3.3.3. Vagotomy during elevated drive

In three additional trials vagal blockade was induced in MK-801 treated animals during exposure to 5% CO_2. Hypercapnia in the MK-801 treated animals resulted in and increase in the number of breaths occurring per episode. Subsequent vagal blockade resulted in a prolonged apnea that was ultimately interrupted by single breaths which occurred with increasing frequency until arousal from hibernation was initiated. In no cases did an episode of breathing occur before or during the arousal process.

7.3.3.4. Additional trials

In four additional trials, MK-801 treatment was administered subsequent to vagal blockade. Vagal blockade depressed ventilation through a decrease in the number of breaths occurring per episode. Injection of MK-801 solution into the CSF was followed by a period of time during which breathing continued in breathing episodes characteristic of vagotomized

animals. After approximately 30 minutes, however, these episodes abruptly ceased and were replaced by single breaths which occurred with increasing frequency until arousal from hibernation was initiated. A gradual conversion from episodic breathing to breathing in single breaths was not observed.

7.4. Discussion

7.4.1 Euthermic animals

As demonstrated previously (chapter 3), vagal feedback was essential for the expression of spontaneous breathing in unanesthetized squirrels. In the present investigation, spontaneous breathing did not occur following vagal blockade, despite pre-treatment with MK-801. These data reaffirm the presence of a strong negative modulation of breathing which is overcome by vagal feedback, and show that this negative modulation does not involve NMDA receptor-mediated processes.

7.4.2 Anesthetized animals

Following vagotomy subsequent to MK-801 treatment, animals exhibited a pattern of intermittent apneusis. Most inspirations were greatly prolonged. These inspirations consisted of two phases. The first phase was associated with inspiratory air flow and lasted approximately the same length of time as a normal inspiration in control animals. During the second phase, measurements indicated that while the inflated state of the chest was actively maintained, inspiratory flow had ceased. This prolonged active inspiration doubled the inspiratory phase duration. These results are consistent with reports of apneusis following MK-801 administration and vagotomy in other mammals (Pierrefiche et al. 1994; Abrahams et al.

1993; Cassus-Soulanis et al. 1995; Fung et al. 1994; Feldman et al. 1992; Connelly et al. 1992; St.John, 1979).

Apneusis and apnea are both periods during which ventilatory flow is absent. During apneusis, however, respiratory muscle tone is maintained while it is absent during apnea. Distinctions between apneusis and apnea are primarily based on direct assessment of phrenic nerve or respiratory muscle activity in artificially ventilated animals, with such activity present during apneusis and absent during apnea (Pierrefiche et al. 1994). End-inspiratory apnea can only result from airway closure following chest expansion. While motor output was not measured in the present investigation, animals were breathing spontaneously via a tracheal cannula which bypassed the glottis and eliminated the possibility of airway closure. Thus, for chest expansion to be maintained during the second phase of inspiration following MK-801 treatment and vagotomy would require muscle activation indicative of apneusis.

In the current study the mean duration of the end-inspiratory pause was 0.34 ±0.07 seconds, although, apneustic pauses lasting 3-5 seconds were not uncommon. On rare occasions, pauses greater than 15 seconds were observed. These durations are consistent with those observed in rats (Montreau et al. 1990; Connelly et al. 1992; Cassus-Soulanis et al. 1995) but are considerably shorter than those observed in cats (Foutz et al. 1988; Pierefiche et al. 1994; Feldman et al. 1992; St.John, 1979).

Hypercapnic exposure resulted in increases in ventilation, mediated by an increase in tidal volume due to a further increase in inspiratory duration. This observation illustrates that the apneusis resulting from MK-801 was not a prolonged inspiration to total vital capacity, limited by mechanical properties of the lung and chest. It suggests that these apneustic inspirations occur via a more controlled process. Stella (1938) first demonstrated that tidal

volume could be increased by hypercapnia during apneusis, and many subsequent studies have confirmed this observation (Von Euler et al. 1976; St.John, 1979; Pierrefiche et al. 1994).

Apneustic breathing, thus appears to result from the prolonged activation of inspiratory motor nerves and associated muscle fibers (motor-units). The vagal and pontine inspiratory-termination mechanisms are not active and, thus, inspiration is prolonged until other mechanisms, not normally involved in inspiratory-termination, stop inspiration (Von Euler et al. 1970; Von Euler and Trippenbach, 1975 and 1976) Inspiratory volume is defined by the number of motor-units recruited. Inspiration proceeds for the length of motor-unit stimulation. The absence of both vagal feedback from pulmonary stretch receptors, and the influence of the PBC, delays the inspiratory off-switching mechanism and produces an abnormal prolongation of inspiratory muscle tone beyond the point where chest expansion is complete for this degree of motor unit recruitment. The inspiratory volume is maintained during the apneusis by continued activation of these contracted muscles which hold the chest in an inflated state. Eventually, inspiratory drive terminates via mechanisms unrelated to vagal feedback or input from the PBC, ending the apneustic event. Following expiration, the process begins anew (Von Euler and Trippenbach, 1975 and 1976).

Hypercapnia results in an increase in ventilation mediated by an increase in tidal volume, with an associated increase in the durations of inspiration. This suggests that a larger pool of inspiratory motor-units are recruited over a slightly longer inspiratory duration. These data indicate that the number of motor units which are recruited is determined by the integration of chemoreceptor inputs, and that this process is unaffected by vagotomy and MK-801 treatment.

7.4.2.3. Apneusis in rodents

The results of the present investigation indicate that MK-801 treatment results in apneusis in vagotomized anesthetized ground squirrels. Cats generally exhibit a profound apneusis following the removal of both the influences of the PBC and vagal afferent feedback. Apneusis, however, has not always been observed when similar treatments were applied to rodents.

Monteau et al. (1989) did not observe apneusis following vagotomy and pontine transection in (strain not stated) rats anesthetized with urethane or pentobarbital. They concluded that there were interspecific differences between cats and rats in the pontine influences on the medullary respiratory generator. In a subsequent study, however, Monteau et al. (1990) did observe a prolongation of inspiration following intravenous administration of MK-801 in vagotomized paralyzed (strain not stated) rats anesthetized with pentobarbital. Drastically prolonged "apneusis-like" inspirations occurred in 4 of their 11 experiments. They concluded that apneusis was difficult but possible to obtain in rats.

As the results of Monteau et al. (1990) did not agree with some of their preliminary findings, Connelly et al. (1992) investigated the influences of MK-801 and vagotomy on rats anesthetized with chloral hydrate. These authors obtained apneusis in 60% of the Sprague-Dawley rats, but not in Wistar rats that they tested. They concluded that there were differences in the respiratory responses to MK-801 indicative of differences in respiratory control mechanisms between these two rat strains.

Wang et al. (1993) obtained apneusis in vagotomized paralyzed Sprague-Dawley rats during combined urethane and chloralose anesthesia, following electrolytic lesion of the PBC. Soon after this, Fung et al. (1994) determined that unilateral microinjection of MK-801 into the

PBC of decerebrate vagotomized paralyzed Sprague-Dawley rats prolonged inspiration. The results of these two studies indicate that the brainstem respiratory control system is similarly organized in Sprague-Dawley rats and other mammalian species.

Cassus-Soulanis et al. (1995) observed apneusis following systemic MK-801 treatment and vagotomy during anesthesia in mice, guinea pigs and both Sprague-Dawley and Wistar rats. These authors determined, however, that the apneusis disappeared during either deep anesthesia or when anesthetic influences had dissipated. These results also argue against species and strain-specific differences in ventilatory control in rodents. These authors, however, demonstrate that wakefulness maintains normal respiratory patterns despite suppression of both vagal and NMDA-receptor mediated inspiratory off-switch mechanisms, and that anesthetic dose can have a profound influence on experimental results as anesthesia suppresses apneustic inspiratory pauses in rodents.

The results of the present investigation indicate that urethane anesthetized ground squirrels also exhibit apneusis in response to MK-801 treatment and vagotomy. They add to the growing body of literature that suggest the brainstem respiratory control system is similarly organized in all mammals.

7.4.3 Hibernating animals

Previous studies indicated that although either removal of vagal feedback or the functional antagonism of NMDA receptors influenced the pattern of episodic breathing in hibernating animals, neither treatment alone prevented the expression of breathing episodes (chapters 3 and 5). The two together, however, had a profound influence on breathing pattern and did eliminate breathing in episodes. The influence of this combined treatment was difficult

to assess as it also prompted arousal from hibernation. Combined treatment did result in a loss of breathing episodes, suggesting that interaction between vagal feedback and NMDA receptor-mediated processes was responsible for the clustering of breaths into episodes. The gradual increase in the frequency of single breaths which occurred following combined treatment demonstrated that a steady-state breathing pattern did not occur before arousal from hibernation was initiated.

Arousal is problematic in that it introduces a possible confounding factor; are the changes in breathing observed the direct result of the treatment *per se*, or does the treatment induce arousal from hibernation which has a secondary influence on breathing pattern ? MK-801 on its own will produce arousal from hibernation (Harris and Milsom, in preparation). This generally occurs following relatively high doses of MK-801 (5 mg/kg), yet it did occur following the doses used during the present study. Breathing pattern, however, changed well before signs of arousal such as tachycardia and shivering were observed. Furthermore, during spontaneous arousal, breathing typically reverts from episodic to continuous through a transition phase (Fig. 7.5). During this transition, f_R fluctuates such that breathing waxes and wanes appreciably (Milsom et al. In press). Also during a normal arousal, tidal volume initially increases from that observed during hibernation (Harris and Milsom, 1994). When animals aroused following vagal blockade subsequent to MK-801 treatment, breathing episodes were directly replaced by single breaths. During the arousal phase, the frequency of single breaths increased, but waxing and waning was not observed and both breathing frequency and tidal volume remained relatively constant over the period of observation. Thus, during this period breathing is considerably different than that observed during a normal spontaneous arousal further suggesting that even though the total level of ventilation may be a secondary influence

of arousal from hibernation, the change in breathing pattern is not.

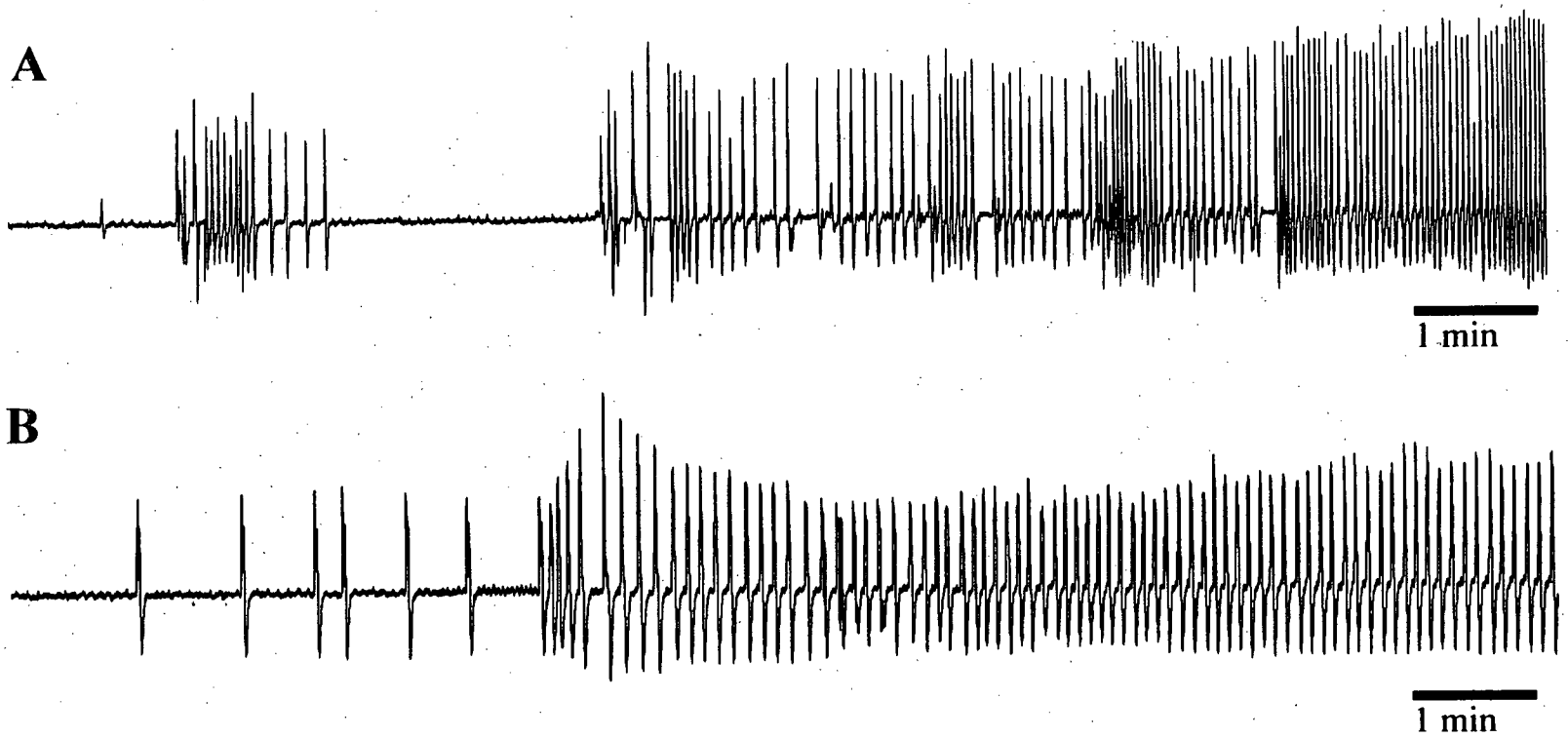
Consequently, these data suggest that one or other of vagal feedback, or glutamatergic input utilizing NMDA type receptors (at an unknown location) are required for the expression of breathing episodes. These episodes do not occur in their combined absence.

During hibernation, golden-mantled ground squirrels exposed to ambient temperatures ranging approximately between 5 and 10 °C normally breath in distinct episodes. If ambient temperatures are decreased to 2 °C, the animals body temperature falls below an apparent critical threshold (between 6-7 °C,) and breathing episodes are replaced by evenly spaced single breaths, although f_R and V_E are unchanged (Milsom, 1988). In addition, squirrels hibernating at an ambient temperature of 5 °C quickly replace breathing in episodes with evenly spaced single breaths following exposure to 1% halothane in air, without any change in body temperature (Milsom et al. In press). These data give rise to the hypothesis that breathing in episodes in generated by an influence from supramedullary structures that are removed through cooling or anesthesia. Removal of this influence results in a breathing pattern of evenly spaced breaths (Milsom, 1988; Milsom et al. In press). The data from the present study are not inconsistent with this hypothesis. They would suggest, however, that the supramedullary structure must exert its influence via glutamatergic processes utilizing NMDA-type receptors, and that extreme cold or anesthesia in hibernation must also remove vagal influences on the control of breathing episodes.

Apneusis was never observed in hibernating animals. Cassus-Soulanis et al. (1995) have shown that "wakefulness", or the dissipation of anesthesia, maintains a normal respiratory pattern despite the suppression of both NMDA receptor-mediated and vagal-mediated inspiratory off-switch mechanisms. During hibernation, squirrels demonstrate profound

Fig. 7.5

Representative recordings of breathing from hibernating squirrels during A) the initiation of a spontaneous arousal from hibernation, and B) an arousal stimulated by vagal blockade subsequent to MK-801 treatment. Breathing records are the direct tracings from the pneumotachograph, with inspiration as an upward deflection and tidal volume proportional to the area under the deflection.


A
1 min
B
1 min

reductions in central activation yet preserve much autonomic function. As the hibernating animals used in this study were not anesthetized, they may still possess the aspects of 'wakefulness" which preclude the expression of apneusis.

7.4.3.1. Vagotomy during elevated drive

Vagotomy itself reduces the number of breaths occurring within a breathing episode (chapter 3). It is possible that the resulting pattern of single breaths following vagotomy subsequent to MK-801 treatment reflects the production of breathing episodes, each containing only one breath. As hypercapnia produced increases in the number of breaths occurring in each episode following either vagal blockade or MK-801 treatment alone, it was predicted that if each breath constituted an episode then hypercapnic exposure could stimulate multi-breath episodes following combined treatment. The results indicate that even at elevated levels of ventilatory drive, breathing episodes do not occur following combined treatment.

7.4.3.2 The nature of vagal feedback in hibernation

Vagal input in anesthetized euthermic squirrels stimulates breathing by facilitating breathing frequency. Vagal input alone in hibernating animals continues to stimulate breathing by facilitating overall breathing frequency. Removal of vagal feedback subsequent to MK-801 treatment initially results in a prolonged apnea, again suggesting that vagal feedback stimulates breathing. This combined treatment converts episodes to single breaths. During breathing episodes, each breath contains a short end-expiratory pause, while there are longer end-expiratory pauses between breaths in the single-breath breathing pattern. This again suggests that vagal input is excitatory. The long apneas between episodes, however, are replaced by

shorter pauses between every breath suggesting that vagal input is somehow inhibitory. This last effect, and the effect of combined treatment on overall frequency are complicated since episodes are converted to occasional single breaths which become more frequent over time. Overall frequency is initially reduced by removal of vagal feedback subsequent to MK-801 treatment but as animals approach or begin to arouse, overall breathing frequency is elevated. The possibility that the role of vagal feedback may have become inhibitory is intriguing. It is tenuous, however, to base such a conclusion on the evidence at hand.

7.5. Conclusion

Vagal feedback and NMDA receptor-mediated processes both influence breathing pattern in ground squirrels. The two, however, do not constitute redundant mechanisms of control. In general, vagal feedback stimulates breathing frequency, while NMDA receptor-mediated processes inhibit breathing frequency. Thus, the two influence breathing pattern in different ways. Ironically, however, either one of these modulatory inputs is sufficient for the expression of a relatively normal breathing pattern, while the loss of both greatly disrupts breathing.

In unanesthetized euthermic squirrels vagal feedback is essential for the expression of spontaneous breathing, such that determining the respiratory influences of NMDA receptor antagonism in the absence of vagal feedback is not possible. In anesthetized euthermic squirrels the loss of both vagal feedback and NMDA receptor-mediated processes result in apneusis. Thus, despite the inhibition of breathing frequency by NMDA receptor-mediated processes, which appears unique to this species, NMDA receptor-mediated and vagal feedback-mediated inspiratory off-switch mechanisms appear to interact in the same way in squirrels as

in other mammals.

During hibernation the loss of both vagal feedback and NMDA receptor-mediated processes result in a breathing pattern of relatively evenly spaced single breaths and subsequent arousal from hibernation. This combined treatment results in a disruption of breathing which is greater than that following the loss of either one alone. The combined effects of the two are synergistic and can not be considered as the additive effect of one plus the other. Although hibernating animals do not exhibit apneusis, inspiration is prolonged and consists of a biphasic airflow indicative of dissociation of diaphragm and intercostal inspiratory muscles. The factor responsible for the stimulation of arousal from hibernation is unknown.

The results of this study indicate that while vagal feedback and NMDA receptor-mediated processes are important in shaping breathing pattern in this species in both euthermia and hibernation, their roles are drastically transformed between the two states.

Chapter 8

The Effect of N-methyl-D-aspartate Receptor Blockade in the Pontine Pneumotaxic Center on Breathing Pattern in Ground Squirrels

Abstract

The influences of microinjections of (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-80, a non-competitive antagonist of N-methyl-D-aspartate (NMDA) type glutamate receptors) bilaterally into the Parabrachial complex (PBC), on breathing pattern were assessed in anesthetized, vagotomized, spontaneously breathing golden-mantled ground squirrels. Bilateral pressure micro-injection of MK-801 into the PBC area resulted in apneusis. Microinjections outside the PBC area had no influence on breathing pattern. Exposure to hypercapnia (5 % CO_2) following MK-801 treatment resulted in an increase in tidal volume, while hypoxia (10 % O_2) resulted in an increase in breathing frequency although neither treatment produced an increase in total ventilation. These results indicate that the NMDA receptor-mediated processes within the PBC normally terminate inspiration and prevent the expression of apneusis in vagotomized squirrels. These results also demonstrate that NMDA receptors within the PBC do not influence ventilatory chemoresponses, suggesting that the alteration of chemoresponses observed following systemic MK-801 treatment was due to the disruption of NMDA receptor-mediated processes located outside the PBC.

8.1. Introduction

Apneusis is an aberrant breathing pattern characterized by a drastic prolongation of the inspiratory phase of the respiratory cycle. Apneusis is reliably produced by lesions or transections which deactivate or remove a region of the pons in animals following vagotomy. This critical region is located in the rostral dorsolateral pons and contains the medial and lateral Parabrachial and Kölliker-Fuse nuclei (the Parabrachial complex, PBC). In the rat, the PBC is shown to contain a high concentration of N-methyl-D-aspartate (NMDA) type glutamate receptors, which are relatively scarce in other brainstem regions (Monaghan and Cotman, 1985; Petralia et al.1994). The non-competitive NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801) induces apneusis in vagotomized animals when administered systemically or micro-injected into the PBC (Ling et al. 1994, Fung et al.1994; Cassus-Soulanis et al. 1995). This suggests that the PBC influences breathing pattern through NMDA receptor-mediated processes, and that NMDA receptors outside the PBC are relatively unimportant in the control of breathing pattern.

Systemic application of MK-801 induced apneusis in vagotomized golden-mantled ground squirrels (*Spermophilus lateralis*) (chapter 7). This treatment also eliminated the ventilatory response to hypoxia both before and after vagotomy. The present investigation was designed to test the hypothesis that NMDA receptors located within the PBC are responsible for the apneusis observed following systemic MK-801 treatment. It was designed to also assess whether NMDA receptors within the PBC influenced ventilatory chemoresponses, or whether the alteration of chemoresponses observed following systemic MK-801 treatment were due to NMDA receptors located outside the PBC.

8.2. Methods

8.2.1 Experimental animals

Experiments were performed on a total of seven adult male golden mantled ground squirrels *(Spermophilus lateralis*, 213.2 ± 7.2 g), selected from the colony of research animals described in chapter 2.

8.2.2 Surgical procedure

Animals were anesthetized with a 5ml/kg intra peritoneal injection of a 20% solution of Urethane (Sigma, dose =1g/kg) in saline, and allowed to stabilize for one hour. Supplemental anesthesia, to facilitate surgery, was induced using vaporous halothane (Wyeth-Ayerst, 3.5% in air) administered via a tight fitting mask. A ventral incision was made in the neck, the trachea was cannulated, and the left and right cervical vagi were isolated and severed. The tracheal catheter was connected, in series, to a ventilatory pneumotachograph attached to the side arm of a gas line and animals were supplied with room air supplemented with 100% O_2 to produce a final mixture that was 35 ± 2.5 % O_2. Body wall movements associated with ventilation were monitored by respiratory impedance via two silver electrodes (Grass Instruments) secured to shaved patches of bare skin, in opposition across the lateral abdomen. As animals were spontaneously ventilating through a tracheal catheter, any pause in air flow occurring at end-inspiration, with the chest wall expanded, would require respiratory muscle activation, since glottal trapping or breath holding could not occur. Consequently, such a combination would be indicative of a maintained inspiratory effort.

Animals were placed on a servo-controlled homeothermic table (Harvard) to maintain body (rectal) temperature at 36 ± 1 °C throughout the experiment, and were then secured in a

stereotaxic head frame (Kopf). Pilot investigations indicated that the skull geometry of ground squirrels was slightly different from that of rats. When squirrels were secured in a stereotaxic head frame with the bite-bar set to +5.0 mm, however, regions of the caudal brain could be located reliably using anterior-posterior and dorsal-ventral stereotaxic coordinates established for the rat (Paxinos and Watson, 1986) after rotating the coordinate axes -28° about the interaural line.

Four cranial electroencephalographic (EEG), two electrocardiographic (ECG), and two electromyographic (EMG) electrodes were implanted in the skull, rib-cage and shoulder musculature, respectively as described in chapter 2.

An access hole was drilled in the cranium spanning the area between 0.5 to 3.5 mm caudal to the intra-aural line and 2.5 mm to either side of the mid-sagittal suture, using a miniature drill (Dremmel) mounted in a stereotaxic micro-manipulator. Care was taken to leave the underlying dura intact, and this was moistened with regular applications of mineral oil and/or isotonic saline.

8.2.3 Microinjection apparatus

Multi-barrel glass micropipettes (35-40 μm outer tip diameter) were fabricated from five-barrel microfilament capillary glass (1.2 mm outer diameter, 0.6 mm inner diameter, AM Systems) using a Narashige pipette puller, and broken back until the total tip diameter measured approximately 35-40 μm. Barrels were two-thirds filled with either isotonic saline (one barrel), MK-801 solution (14.8 mM in isotonic saline, pH = 7.3 ± 0.1, three barrels) or a mixture of pontamine sky-blue dye in isotonic saline (one barrel). Barrels were filled such that each meniscus was visible. A piece of polyethylene tubing (PE 10, Clay Adams) was secured

into the top of each barrel with epoxy adhesive at one end and a manifold constructed of a series of five 3-way stopcocks at the other. The manifold was connected to a pressure ejection system (Picospritzer II, General valve Corp.) such that only one barrel of the multi-barrel micro-pipette was exposed to any given pressure pulse. Both the duration and magnitude of the pressure pulse could be adjusted on the pressure ejector, and the injection volume was calculated from the radius of the micro-pipette barrel and the distance traveled by the meniscus during the injection as measured with a microscope equipped with a fine reticule.

8.2.4 Experimental protocol

The micro-pipette was secured into a 3-dimensional micro-manipulator (Kopf) and positioned over the access hole 2.0 mm to the right of midline and 2.0 mm caudal to the inter-aural line. The meninges were perforated with a hypodermic needle, and the micro-pipette was dropped to approximately 6.8 mm below the surface of the brain. Pilot investigations had indicated that this plane of approach would intersect the area of the PBC at approximately 7.5 mm below the surface of the brain. Each transect began with an injection of approximately 50 nl of saline, using 5 or more pressure pulses of 10 to 50 ms duration. Following a 10 minute pause, the micro- pipette was moved 0.2 mm ventrally and approximately 50 nl of MK-801 solution was injected. The procedure of pausing for 10 minutes, extending the micro-pipette 0.2 mm and injecting MK-801 solution was repeated approximately 8 to 10 times and was followed by a 100 nl injection of dye to mark the bottom of the transect. Pilot studies indicated that dropping the micro-pipette 9.0 mm below the surface of the brain would bring it well beyond the region of the PBC.

The micro-pipette was retracted from the brain and repositioned on the contralateral side

over the access hole 2.0 mm to the left of midline and 2.0 mm caudal to the inter-aural line. The meninges were again perforated with a hypodermic needle, the micro-pipette was dropped to approximately 6.8 mm below the surface of the brain and the injection procedure was repeated.

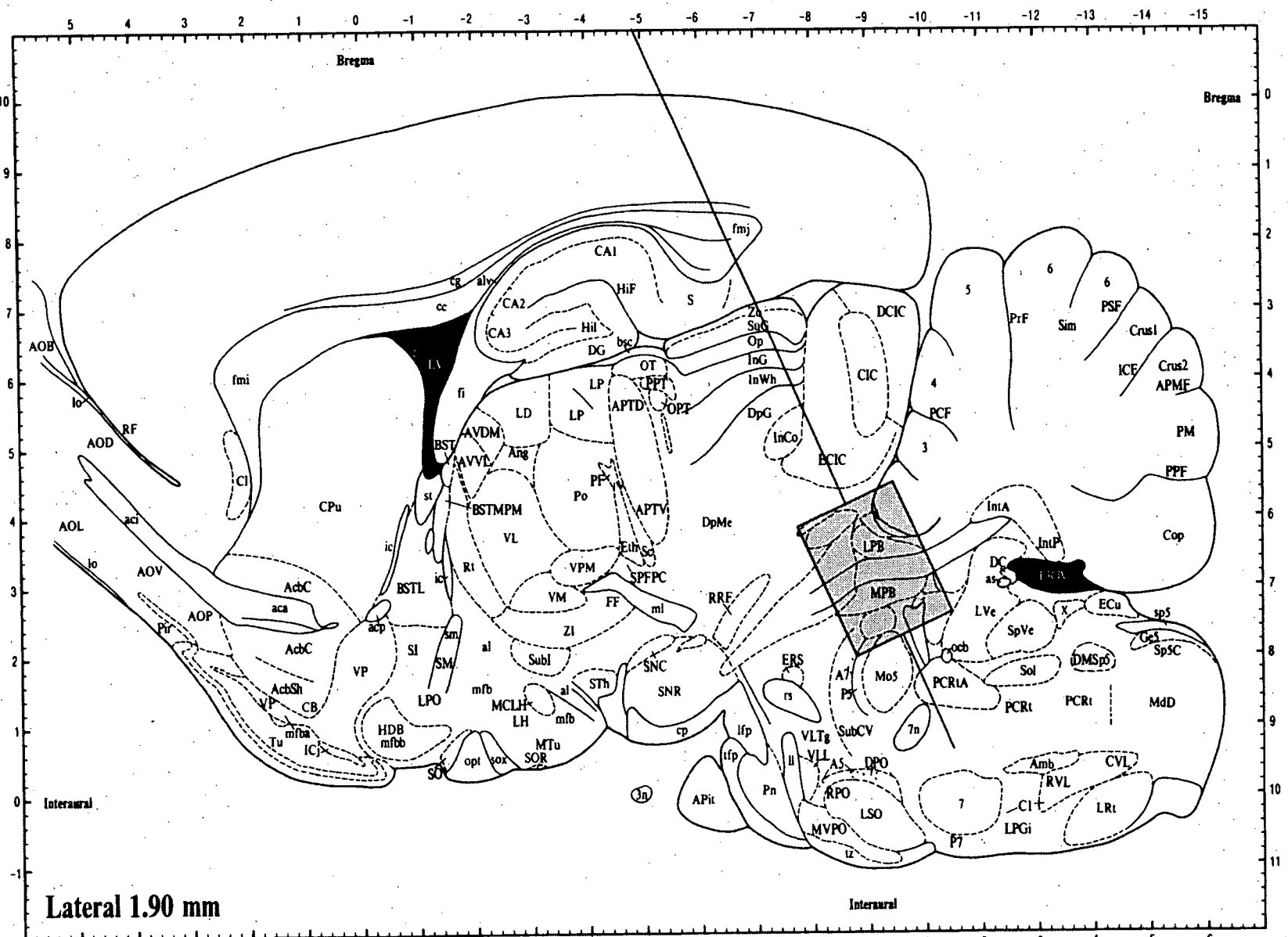
If apneusis was not produced by the first series of bilateral injections, the micro-pipette was retracted from the brain, repositioned over the access hole 0.5 mm ahead of the previous transect and replaced at approximately 6.8 mm below the surface of the brain where the injection procedure was again repeated. This procedure was then repeated at the same coordinates on the contralateral side if necessary. Bilateral transects were then repeated 0.5 mm behind, and then lateral to, the initial transect until either five transects had been made on either side, or until apneusis was observed. Figure 8.1 is a schematic representation of the path of travel of the micro-pipettes during sequential transects and the area targeted by pressure microinjections. Apneusis occurred in five of six animals and required from three to five series of bilateral transects.

Once apneusis occurred, the cite of last injection was marked with a dye injection and the injection protocol was abandoned. The apneustic breathing pattern was monitored for at least 20 minutes, following which animals were exposed to 5% CO_2 in air for 20 minutes. Animals were then returned to air supplemented with 100% O_2 for 20 minutes. Following this they were exposed to 10 % O_2 in N_2 for 20 minutes and then returned to the O_2 supplemented air mixture.

When observations were completed, animals were sacrificed with a 1 cc intra-cardiac injection of sodium pentobarbital (65 mg/ml) and their brains were removed for histological inspection.

Fig. 8.1

An illustration of a sagittal section cut 1.90 mm lateral to the midline through the brain of a rat (Paxino and Watson, 1986). Superimposed on the illustration is a line drawn at -28° to the horizontal plane representing the initial path along which the micro-injection pipette was dropped to locate the pontine respiratory complex. The shaded region represents the area from 7 to 9 mm below the surface of the brain and 0.9 mm anterior and posterior to the initial path influenced by MK-801 microinjections during anterior and posterior transects. The lateral and medial Parabrachial nuclei are labeled as LPB and MPB respectively. The Kölliker-Fuse Nuclei is not shown in this section and is slightly lateral and ventral to the caudal portions of the MPB.



Lateral 1.90 mm

8.2.6 Sham treatment

In a single animal the experimental protocol was repeated, but isotonic saline was substituted for the MK-801 solution. The animals received five series of injections of saline on each side with dye injections marking the top and bottom of each transect.

8.2.7 Data analysis

The EEG and EMG records were scrutinized for evidence of changes in arousal or activation state, based on established criteria for determination of sleep and wakefulness (Reschtaffen et al. 1968).

Ventilation was measured from the integrated output of the tracheal pneumotachograph, using the post-hoc data analysis functions associated with the computer data acquisition system (ADVPOST and WindaQ, DataQ Instruments). Respiratory frequency (f_R), tidal volume (V_T), inspiratory (T_I) and expiratory (T_E) duration, the durations of the periods during which ventilatory airflow was occurring during inspiration (T_{IA}) and expiration (T_{EA}) and the durations of end-ventilatory pauses during inspiration (T_{IP}) and expiration (T_{EP}) were measured from representative 120-second periods of stable breathing. Ventilation (V_E) was calculated as the sum of tidal volumes occurring over each 120 second period divided by 2. The inflation state of the chest was confirmed from the respiratory impedance record and by direct observation. Each of the breathing pattern variables noted above were compared before and after the occurrence of apneusis and during apneustic breathing while exposed to hypoxic and hypercapnic gas mixtures, using a series of one way repeated measures analysis of variance (SigmaStat, Jandel Scientific). Additional pairwise multiple comparison procedures were done using the Student-Newman-Keuls method. All tests employed an alpha value of 0.05, thus,

normality, equality of variance and significance of differences were attributed to $P < 0.05$. Values are reported in the text as mean ± one standard error of the mean.

8.2.8 Histological inspection

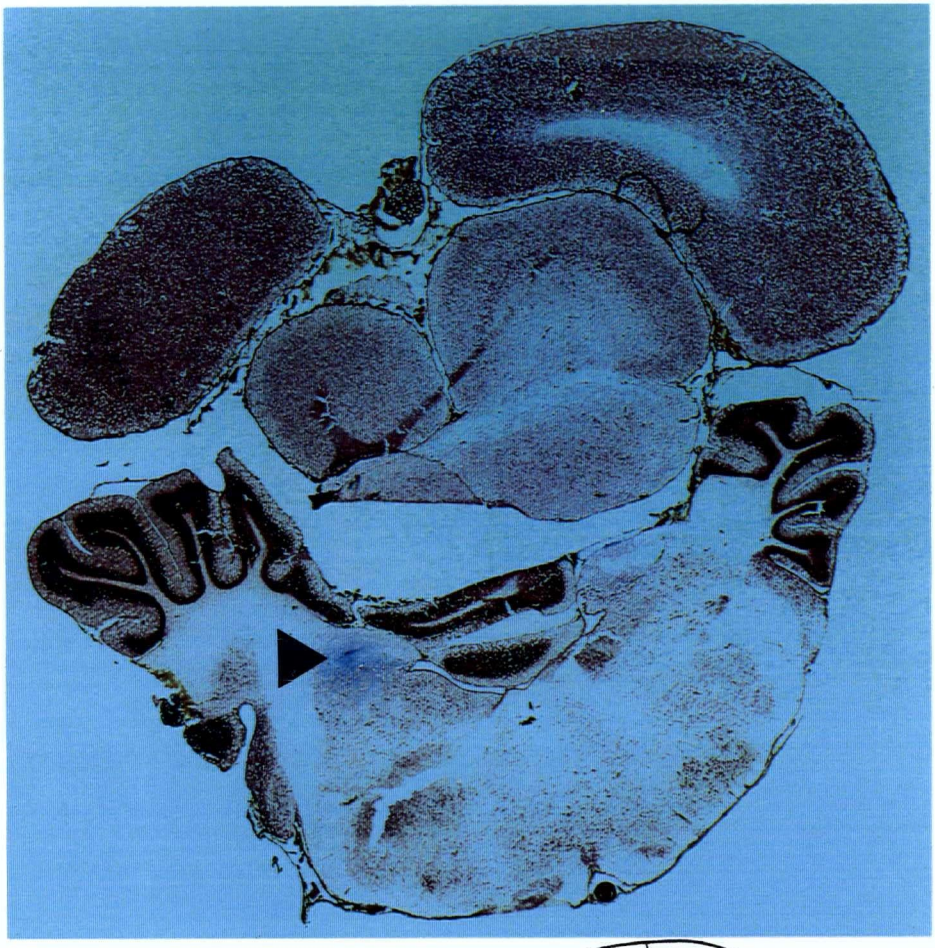
Following each experiment the brain was removed and fixed by 24 hour immersion in Pease's fixative, a solution of 4 % paraformaldehyde in phosphate buffer (Hockfield et al. 1993). Tissues were cryoprotected by 24 hour emersion in 12, 16 and 22 % sucrose solutions in phosphate buffered saline (Hockfield et al. 1993), and frozen into blocks of embedding media (tissue-tek, Miles laboratories), in a column of dry-ice vapor.

Coronal sections (20 μm) were cut through the brainstem on a freezing microtome and mounted to frosted glass slides. Sections were counter stained with neutral red stain (1% neutral red solution in distilled water, with 4% acetate buffer solution (2 parts 0.1 N acetic acid to 3 parts sodium acetate), washed with distilled water, dehydrated by emersion in 70, 95, and 100 % ethanol, permeated with Xylene, and set beneath cover slips with mounting media (Permount, Sigma). Sections were viewed under a light microscope and dye markers indicating the bottom of each transect and the position of last injection before the occurrence of apneusis were localized to approximate the areas influenced by MK-801 injection (Fig. 8.2). Sections were compared against drawings of coronal sections taken from a rat stereotaxic atlas (Paxinos and Watson, 1986). Anatomical landmarks used to determine the location of the PBC were the caudal portions of the cerebral cortex, the fourth ventricle, the cortex of the inferior colliculus, and the paraflocculus and simple lobules of the cerebellum.

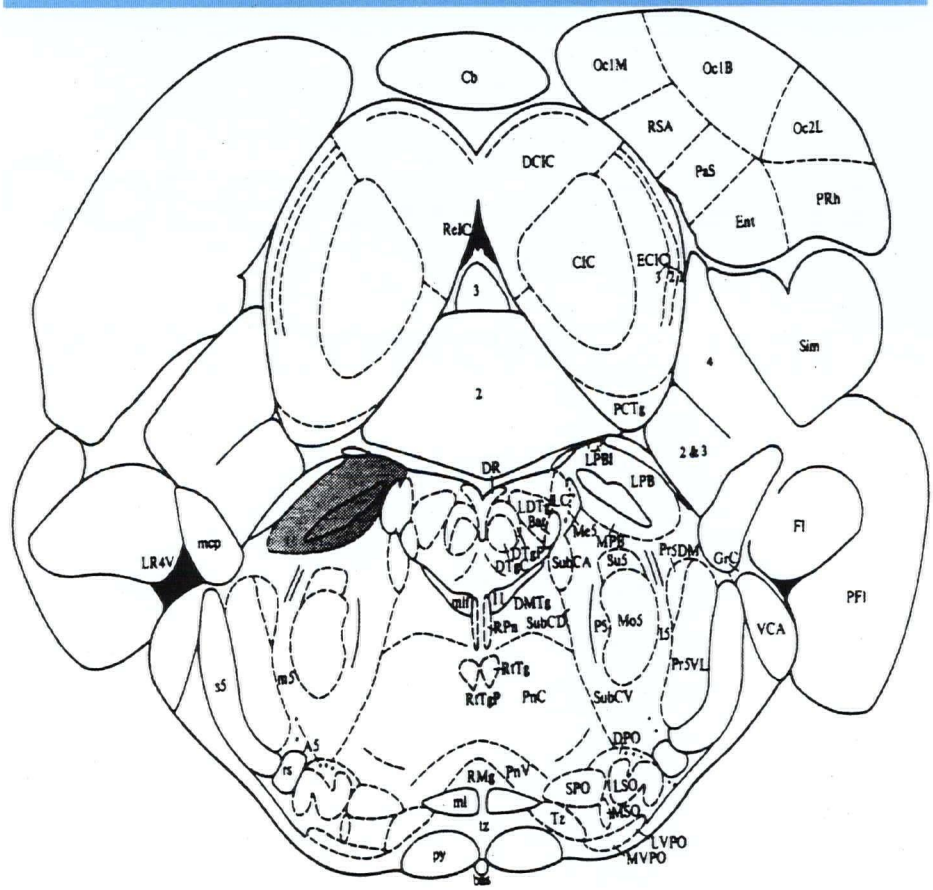
Fig. 8.2

A) A coronal section through the rostral brainstem approximately 0.3 mm caudal to the interaural line. A blue dye spot, indicating the location of an MK-801 injection which produced apneusis, is noted by the arrow. B) Schematic diagram of a similar section taken from a rat stereotaxic atlas (Paxino and Watson, 1986). The target area (the PBC) is shaded on the illustration and the lateral and medial Parabrachial nuclei are labeled as LPB and MPB respectively. The Kölliker-Fuse nucleus is not shown and is slightly lateral and ventral to the MPB and approximately 0.5 mm caudal to this section. Histological inspection indicated that injections producing apneusis all included the PBC area while injections outside the PBC had no effect.

A)



B)


Cb
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DR
LPBl
LPB
LDTg
LC
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Me5
MPB
SuS
PrSDM
GrC
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VCA
PrSVL
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DPO
RMg
PnV
SPO
LSO
MSO
LVPO
MVPO
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8.3. Results

8.3.1. Initial values

In the present study, animals were vagotomized during the initial surgical procedure. As a consequence the initial values measured in this study were for vagotomized animals. Breathing following vagotomy in the present investigation was similar to that observed following vagotomy in the investigation outlined in chapter 7. As it is more reveling to compare the breathing pattern during apneusis with eupnea, values representing eupnea were taken from this previous investigation and are included as "control" values in the current analysis. In the present investigation, vagotomized animals exhibited higher tidal volumes and longer periods of inspiration and expiration than these "control" values.

Most of the anesthetized animals held in the stereotaxic frame exhibited a consistent EEG waveform which had a predominance of high frequency (>4Hz) low amplitude activity. Previous investigations have demonstrated that this pattern represents an awake-like cortical state (State I) and is commonly observed during anesthesia (Grahn et al. 1989, Hunter and Milsom, 1997; chapter 3). Three of the animals also exhibited periods during which the EEG waveform contained slow-wave-sleep-like (State III) activity. This state, too, commonly occurs during anesthesia but did not occur as frequently in the present study as it had during previous investigations (Hunter and Milsom, 1997; chapter 3). Due to this infrequent appearance, breathing during State III was not quantified in the present study.

8.3.2 Anatomic localization

In the sham treated animal and the five animals in which apneusis was observed, the blue dye deposited at the bottom of each transect was visible slightly below (< 1000 μm) and

caudal (< 500 μm)to the lateral (LPB) and medial (MPB) parabrachial and Kölliker-Fuse (KF) nuclei, and the dye marker deposited at the point of last injection once apneusis was observed was always within this complex (Fig. 8.2). In the single animal which did not exhibit apneusis, the dye marks deposited at the bottom of each transect were all above and caudal to the area of the PBC. It should be noted that pontamine sky blue, as a vital dye, is taken into living cells. The neutral red counter staining procedure leached the pontamine sky blue dye from the extracellular fluid but it remained visible within the cells at the injection site. Before counter staining, however, this marker dye was present over an area 4 to 5 times larger than that depicted in figure 8.2. Thus, it is reasonable to assume that the MK-801 solution also influenced cells beyond the area marked by the remaining dye.

8.3.3 Apneusis

Injection of saline at the top of each transect in experimental animals did not produce any noticeable change in breathing pattern. The single sham treated animal did not exhibit any noticeable changes in breathing pattern over the course of the injection protocol.

Apneusis occurred in five of the six animals injected with MK-801. In these animals, the generation of apneustic breathing resulted in a non-significant depression of ventilation and significantly altered f_R and V_T compared to control values during eupnea. Apneusis was characterized by the development of a pause (T_{IP}, 1.29± 0.33 seconds) following inspiration during which inspiratory flow ceased while the chest remained inflated (Fig 8.3). Figure 8.3 illustrates the dramatic increase in peak inspiratory flow associated with apneusis. Flow then rapidly slows and, despite the maintained chest expansion, there is a slight reversal of flow. In all cases, chest expansion throughout this end-inspiratory pause could be verified by visual

inspection of the animals. This pause was followed by a deflation of the chest and a sudden peak in expiratory flow. Compared to the control condition, there was also a lengthening of the end-expiratory pause (TEP) (Fig. 8.4). VT, TIA, and TEA were all increased by the development of apneusis, while mean inspiratory and expiratory flow rates remained constant. The prolongation of TIA, TEA and TEP, and the appearance of the end-inspiratory pause produced an increase in the duration of the total respiratory cycle (Fig. 8.5) and a decrease in fR (Fig. 8.4).

The development of the end-inspiratory pause was the only difference in breathing pattern observed during apneusis that was not present following vagotomy alone. The mean inspiratory flow rate, TIA, TEA and TEP were all not significantly different than those which occurred following vagotomy. The end-inspiratory pause did, however, prolonge the total ventilatory cycle, yet resulted in only non-significant reductions in fR and VE.

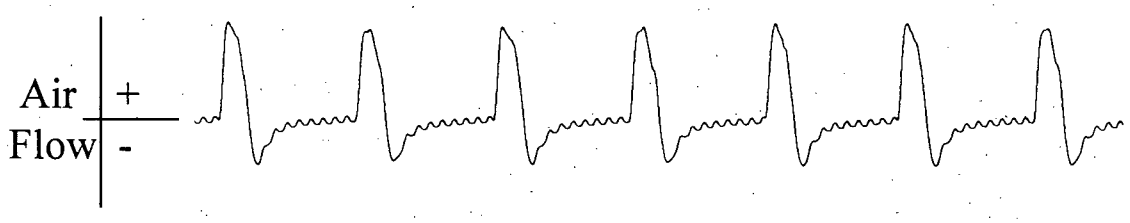
8.3.4 Hypoxia

Exposure to hypoxia during apneusis resulted in a 34 % increase in fR (from 19.5 ± 4.2 to 26.1 ± 5.6 breaths per minute) (Fig. 8.5, 6). This increase was countered by a non-significant decrease in VT, resulting in no changes in VE. Breathing frequency was elevated by decreases in TIA, TEA and TEP, while the duration of the end-inspiratory pause (TIP) remained constant (Fig. 8.4). Prolonged exposure to hypoxia resulted in the occurrence of an epileptiform EEG which returned to normal following a return to breathing O_2 supplemented air.

Fig. 8.3

Breathing pattern exhibited by vagotomized ground squirrels before (top) and after (bottom) bilateral microinjection of MK-801 into the region of the medial and lateral Parabrachial and Kölliker-Fuse Nuclei. Tracings are direct records of tracheal airflow. Inspiration is an upward deflection and tidal volume is proportional to the area under the curve.

Pre MK-801



Post MK-801

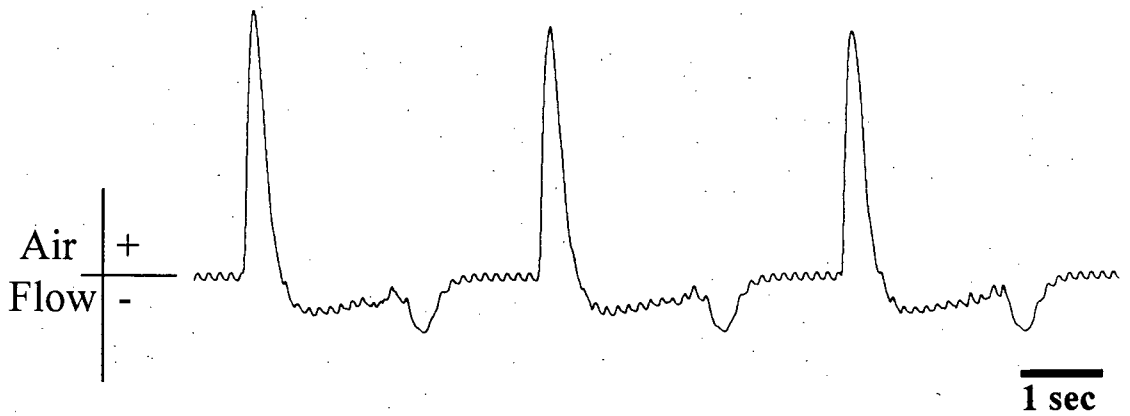


Fig. 8.4

Representation of mean (± standard error) inspiratory volumes and durations of inspiratory flow, the end-inspiratory pause, expiratory flow and the end-expiratory pause in eupnea (◇, Control), following vagotomy (○, VX), and during apneustic breathing during exposure to air (□, air), 10% O_2 (△, Hypoxia) and 5% CO_2 (▽, Hypercapnia). Statistical differences are noted in the text. Note that in different plots the end-expiratory volumes have been offset from zero for clarity.

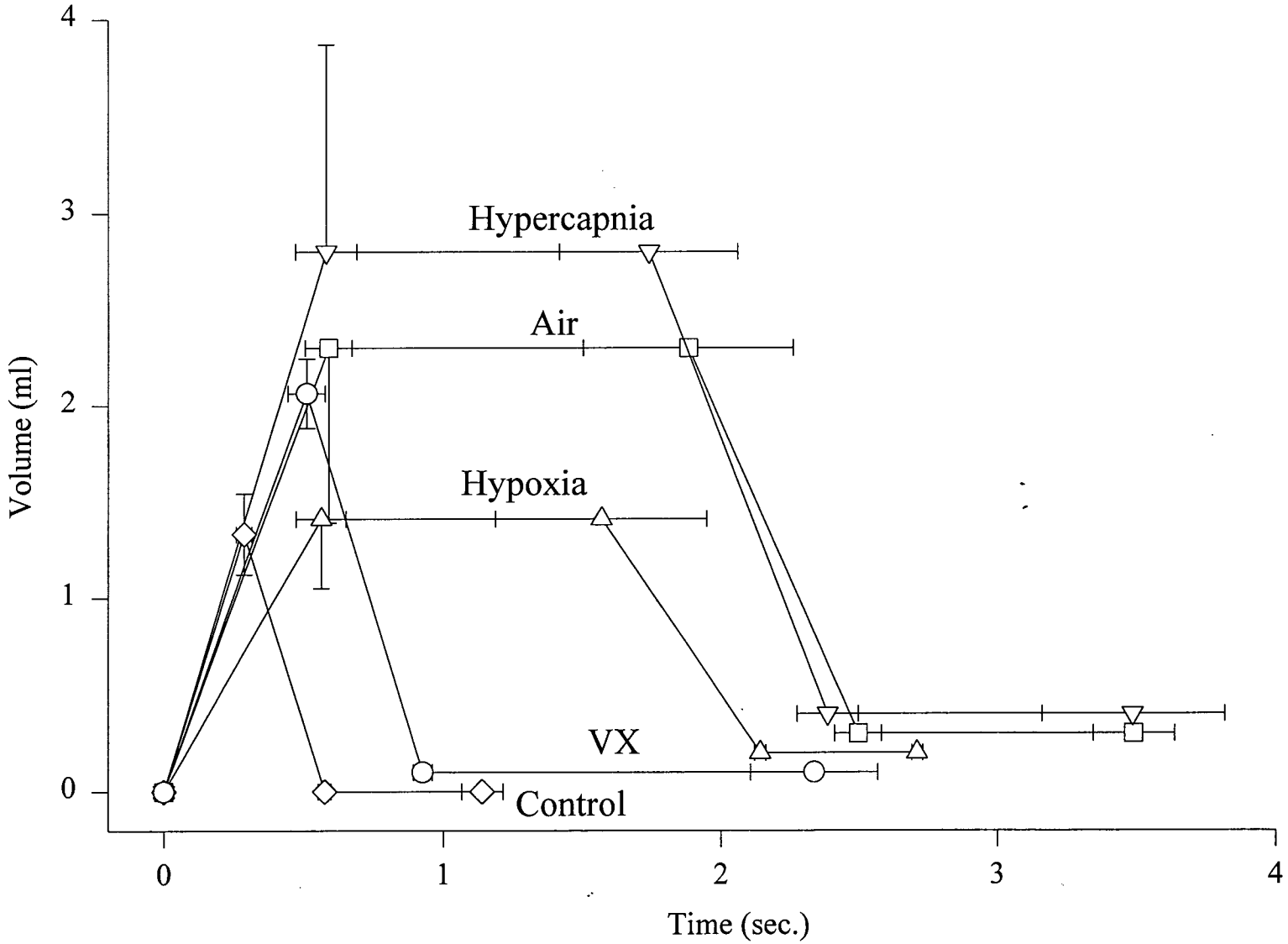

Hypercapnia
Air
Hypoxia
VX
Control
Volume (ml)
Time (sec.)
0
1
2
3
4

Fig. 8.5

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) in eupnea (open bars), and during apneustic breathing during exposure to air (hatched bars, rising right), 10% O_2 (hatched bars, rising left) and 5% CO_2 (crosshatched bars). The "*" denotes a significant difference from the eupnea values, while the "#" denotes a significant difference from apneustic breathing during exposure to air ($P<0.05$).

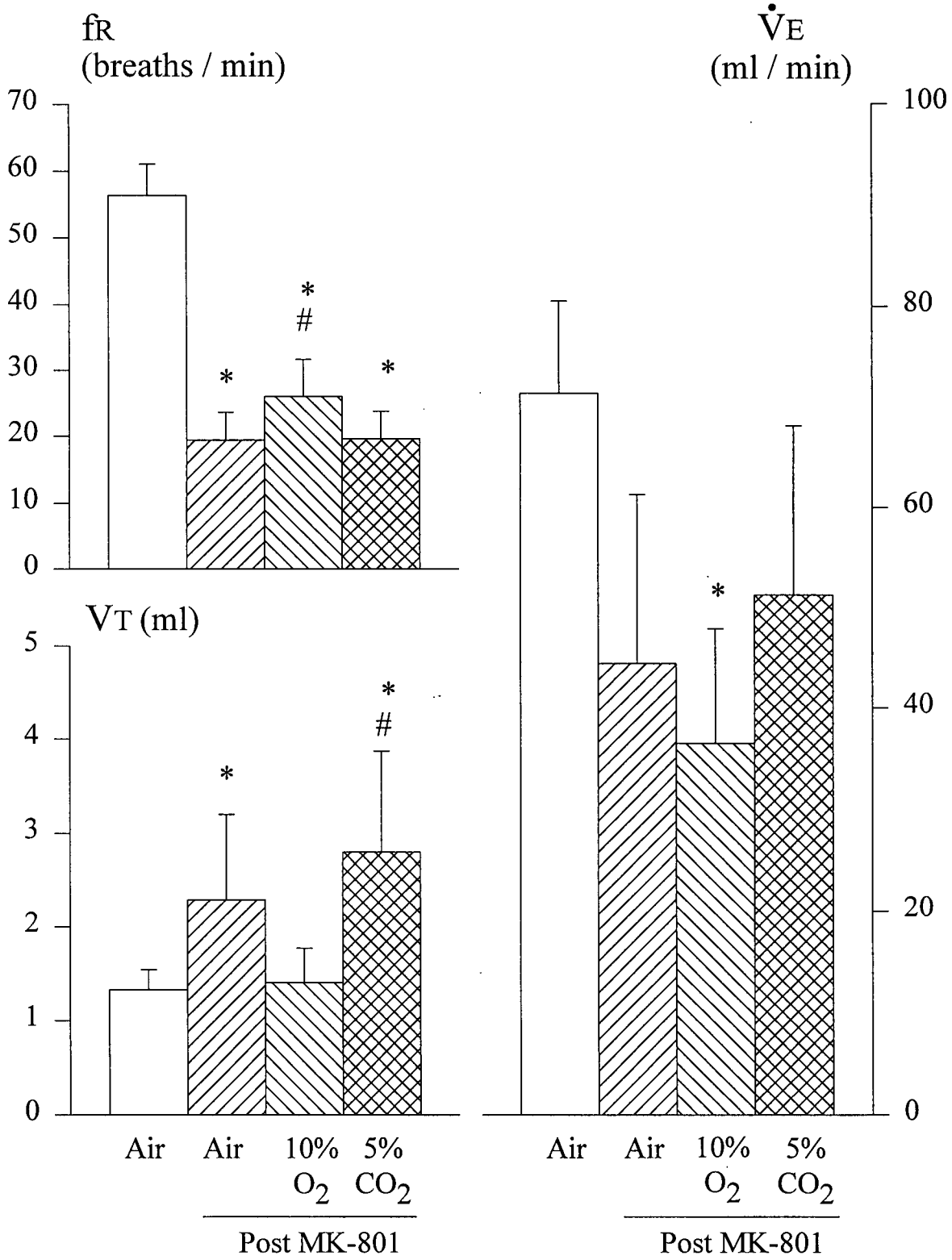
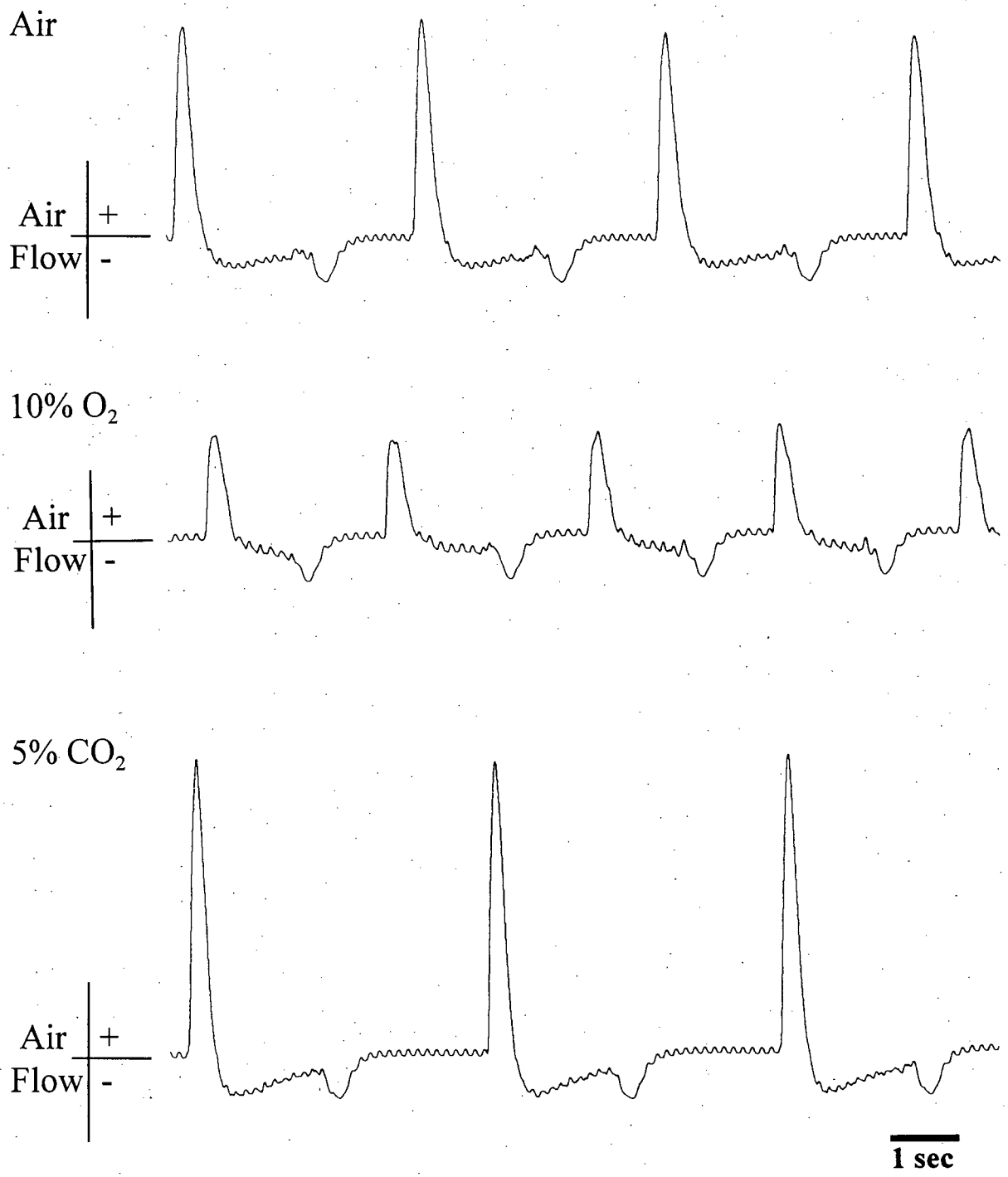

fR
(breaths / min)
70
60
50
40
30
20
10
0
VT (ml)
5
4
3
2
1
0
$\dot{V}_E$
(ml / min)
100
80
60
40
20
0
*
#
Air
Air
10%
O_2
5%
CO_2
Post MK-801
Air
Air
10%
O_2
5%
CO_2
Post MK-801

Fig. 8.6

Breathing pattern exhibited by ground squirrels following bilateral vagotomy and microinjection of MK-801 into the region of the medial and lateral Parabrachial and Kölliker-Fuse Nuclei during exposure to O_2 enriched air (35 ± 2.5% O_2) (top), 10% O_2 (middle) and 5% CO_2 (bottom). Tracings are direct records of tracheal airflow. Inspiration is an upward deflection and tidal volume is proportional to the area under the deflection.


Air
Air
Flow
+
-
10% O_2
Air
Flow
+
-
5% CO_2
Air
Flow
+
-
1 sec

8.3.5 Hypercapnia

Exposure to hypercapnia, during apneusis, resulted in a 22 % increase in V_T (from 2.30 ± 0.91 to 2.80 ± 1.07 breaths per minute) but did not produce significant changes in f_R or V_E (Fig. 8.5, 6). T_{IA}, T_{EA}, T_{EP} and T_{IP} all remained constant during hypercapnia; the increases in V_T were achieved through increases in the mean inspiratory flow rate (Fig. 8.4).

8.4. Discussion

8.4.1. Initial values

For the most part, animals in the present investigation exhibited a consistent pattern of EEG activity which, in previous studies, was designated as State I and taken to represent a state of cortical activation similar to wakefulness (Grahn et al. 1989; Hunter and Milsom, 1997; chapter 3). Some of these animals also exhibited an EEG pattern which has been taken to represent a state homologous to slow-wave sleep (State III, Grahn et al. 1989; Hunter and Milsom, 1997; chapter 3). The latter was only observed in a subset of animals before the initiation of the micro-injection protocol, however, and was never observed after the occurrence of apneusis. The study reported in chapter 3 illustrated that anesthetized squirrels cycled regularly between States I and III, and continued to exhibit State III following vagotomy. Failure to do so by vagotomized animals in the present study was most likely due to the physical nature of the protocol. Previous observations indicate that, during anesthesia, these animals will switch from State III to State I in response to various stimuli including sound, light, and vibration. Thus, the ambient stimuli associated with the experimental procedure or the use of the stereotaxic frame, may have precluded the expression of State III in most animals. Systemic application of MK-801 prevented the expression of State III in this species

(chapter 5). MK-801 micro-injection into the PBC may have further contributed to the absence of State III following this treatment (chapter 5). Since there was so little time spent in State III prior to microinjection, however, this is impossible to assess.

It must also be noted that the breathing pattern of vagotomized squirrels observed during the initial stages of this investigation are not equal to those previously reported for vagotomized squirrels. VT, mean inspiratory flow rate and TEP are all significantly less than those reported in chapter 3 although the level of VE is similar to that reported previously. The reasons for these differences are unknown.

8.4.2 Anatomic localization of microinjections producing apneusis

The brain sections taken from the sham treated animal and the five animals which exhibited apneusis all indicated that the array of injection transects ran approximately through the PBC (Fig 1). Similarly, the dye mark which indicated the final injection site that caused apneusis always included the PBC (Fig. 8.2a, b). Nicholson's (1985) model for the diffusion of neuroactive substances into brain tissue predicts that cells located 200 μm away from a 10 nl injection will be exposed to the equivalent of 35% of the initial concentration of that injection 10 seconds after the injection was made. Given this, 50 nl injections should have been sufficient to influence NMDA receptors within approximately 400 μm of the injection site. The injection volumes and MK-801 concentrations used in the present investigation were well within the range used in similar studies (Ling et al. 1994; Fung et al. 1994; Fung and St.John 1994).

MK-801 is a potent non-competitive antagonist having both a rapid onset and long duration blockade (Huettner and Bean, 1988). It was assumed that the influences of individual

injections would be manifest within the 10 minute observation period between injections and would persist for the duration of the study. In the present investigation, three to five bilateral transects, with ten 50 µl injections per transect were required before apneusis was observed. Thus, these animals received up to 2500 µl of MK-801 solution per side, along three to five 2 mm long columns of cells. This injection protocol would influence cells ranging from 400 µm medial, to 900 µm lateral and 900 µm rostral and caudal to the initial transects (Fig. 8.1, 2).

The histological evaluations confirmed that the MK-801 injections which caused apneusis in these animals occurred within the PBC. It was not possible to determine which of the specific nuclei comprising the PBC was responsible for the induction of apneusis. Fung and St.John (1994) have shown that the Kölliker-Fuse and lateral and medial parabrachial nuclei each have different influences on breathing pattern. Based on kainic acid microinjection into specific regions within the PBC of cats, these authors have proposed that neurons which contribute to the termination of inspiration are located within the rostral portion of the Kölliker-Fuse nucleus and rostral regions of the nucleus parabrachialis lateralis. In the present study, these rostral structures were not within the area targeted by the initial transect, but would have been influenced by fourth and fifth transects. The observation that usually four to five transects were required to produce apneusis, therefore, supports the hypothesis that rostral structures within the PBC contribute to the termination of inspiration while more caudal structures do not. Studies which ascribe specific functions to particular regions of the PBC have been done using larger species (Fung and St.John 1994; Mutolo et al. 1997), or more reduced preparations (Fung et al. 1994), and have involved more precise injection protocols than that presently employed. A similarly detailed characterization in the ground squirrel is beyond the scope of the present investigation.

The position of dye markers in the single animal which did not exhibit apneusis, indicated that injection transects ran well outside the PBC. The data from this animal were not included in any aspect of the analysese. This animal does, however, illustrate that injection of MK-801 solution outside the PBC does not result in apneusis.

8.4.3 Apneustic breathing pattern: Air

Following bilateral micro-injection of MK-801 into the PBC, animals developed the apneustic breathing pattern illustrated in figure 8.3. Apneusis resulted from the development of a pause (TIP) which occurred between TIA and TEA. As these animals were breathing spontaneously via a tracheal cannula which bypassed the glottis and eliminated the possibility of airway closure, and maintained chest expansion throughout this period, these end-inspiratory pauses must have resulted from prolonged activation of inspiratory muscles. Despite the maintenance of chest expansion, there was a slight reversal of flow during the TIP which preceded expiration. It appears, therefore, that despite prolonged activation of the inspiratory muscles the full inspired volume was not retained. This reversal of flow could be attributed to a variety of causes ranging from a decrease in the level of inspiratory activation during apneusis, to muscle adaptation to a constant level of activation. This apneustic inspiration was followed by a deflation of the chest and a sudden peak in expiratory flow (TEA), followed by an end-expiratory pause (TEP) separating the period of expiratory air flow from the next inspiration, during which the chest was relaxed. This pattern of apneusis is akin to that observed following similar treatments in other species (Fung et al. 1994; Ling et al. 1994, Fung and St. John, 1994). These data indicate that functional NMDA receptors located in the PBC actively inhibit apneusis in vagotomized ground squirrels, and that apneusis results from the blockade of these

receptors by MK-801 micro-injected into the PBC.

The apneustic breathing pattern observed in the present investigation differed from that observed following systemic application of MK-801 in vagotomized squirrels (chapter 7, Fig. 8.7). Mean inspiratory flow rates are higher and TIA, TIP and TEP are shorter during apneusis generated by systemic application of MK-801 than during that which results from MK-801 microinjection into the PBC (Fig. 8.8). As a result, fR during the apneusis produced by systemic application of MK-801 (chapter 7) was considerably higher than that observed during apneusis in the present study (Fig. 8.9). These data support the observation that fR is actively inhibited by an NMDA-mediated process described in chapter 5 and indicate that the NMDA receptors which prevent tachypnia are not located within the PBC.

8.4.4 Apneustic breathing pattern: Hypoxia

Apneusis during hypoxic exposure was still characterized by a pause (TIP) which occurred between TIA and TEA. Again, despite the maintenance of chest expansion, there was a slight reversal of flow during the TIP which preceded expiration, followed by a deflation of the chest during TEA, and an end-expiratory pause (TEP) during which the chest was relaxed preceding the next inspiration (Fig. 8.4, 6). Exposure to hypoxia, during apneusis, produced an increase in fR resulting from decreases in TEP (Fig. 8.3, 4). The results of chapter 4 indicate that this species normally responds to hypoxia by increasing fR. These results indicate, therefore, that an alteration of breathing frequency normally associated with exposure hypoxia is preserved following MK-801 micro-injection into the PBC, despite the advent of apneusis. This result is similar to previous observations that both hypoxic and hypercapnic ventilatory responses are preserved during apneusis, initiated by lesions within the PBC and vagotomy

(St.John, 1979) and, thus, indicate that the exhibition of apneusis does not preclude such responses. These results differ from those obtained following systemic MK-801 treatment with or without vagotomy, both of which abolished the hypoxic ventilatory response (chapter 6 & 7, Fig .8, 9).

Fig. 8.7

Apneustic breathing patterns exhibited by vagotomized squirrels after bilateral microinjection of MK-801 into the region of the medial and lateral Parabrachial and Kölliker-Fuse Nuclei (top), and intravenous administration of MK-801(bottom). Tracings are the integrated output from the pneumotachograph. Inspiration is an upward deflection and tidal volume is proportional to the height of the deflection.

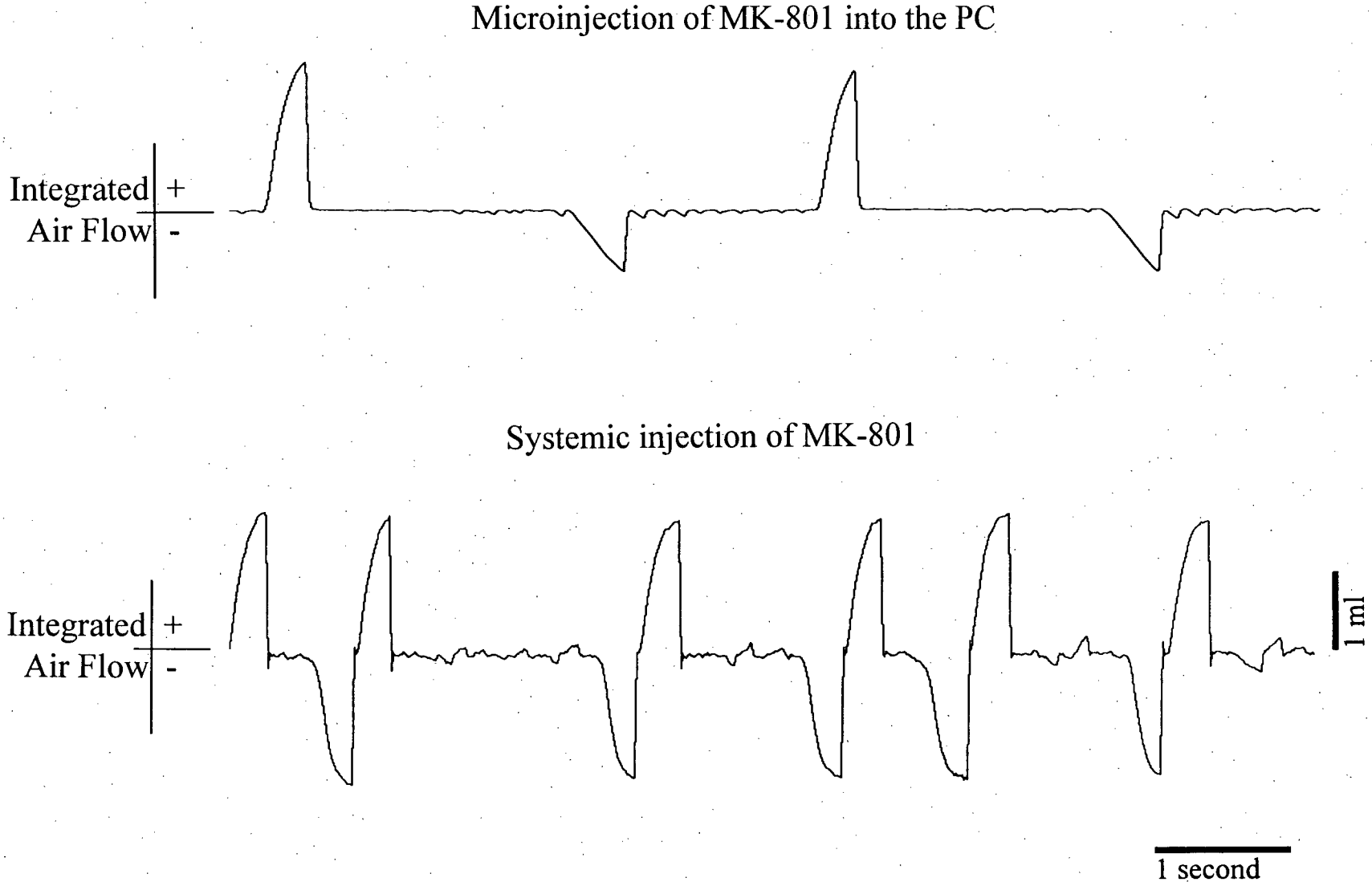

Microinjection of MK-801 into the PC
Integrated Air Flow
+
-
Systemic injection of MK-801
Integrated Air Flow
+
-
1 ml
1 second

Fig. 8.8

Representation of mean (± standard error) inspiratory volumes and durations of inspiratory flow, the post-inspiratory pause, expiratory flow and the post-expiratory pause in vagotomized squirrels, during exposure to air, 10% O_2 and 5% CO_2, after either bilateral microinjection of MK-801 into the region of the medial and lateral Parabrachial and Kölliker-Fuse Nuclei (○,), or intravenous administration of MK-801 (□).

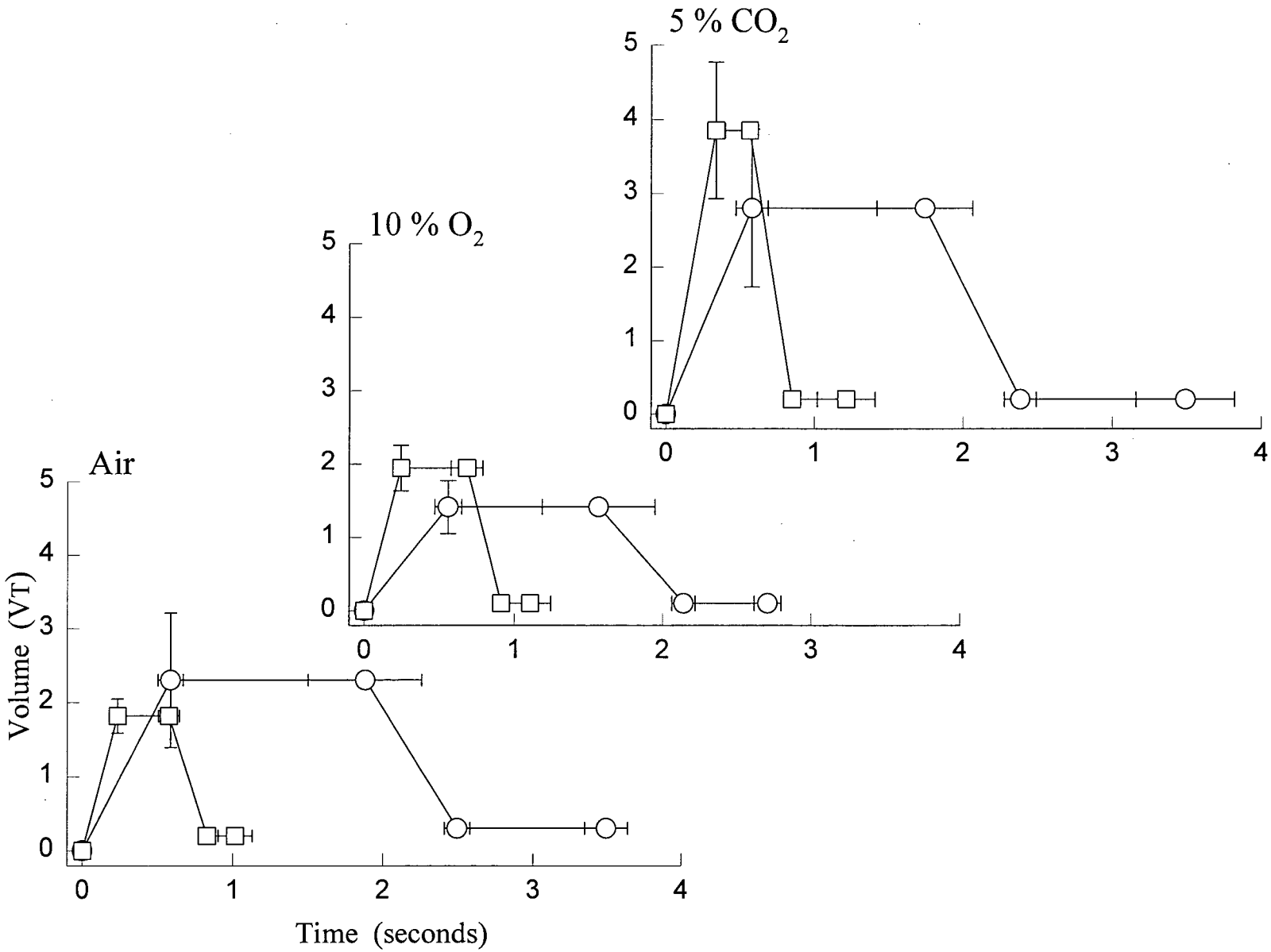
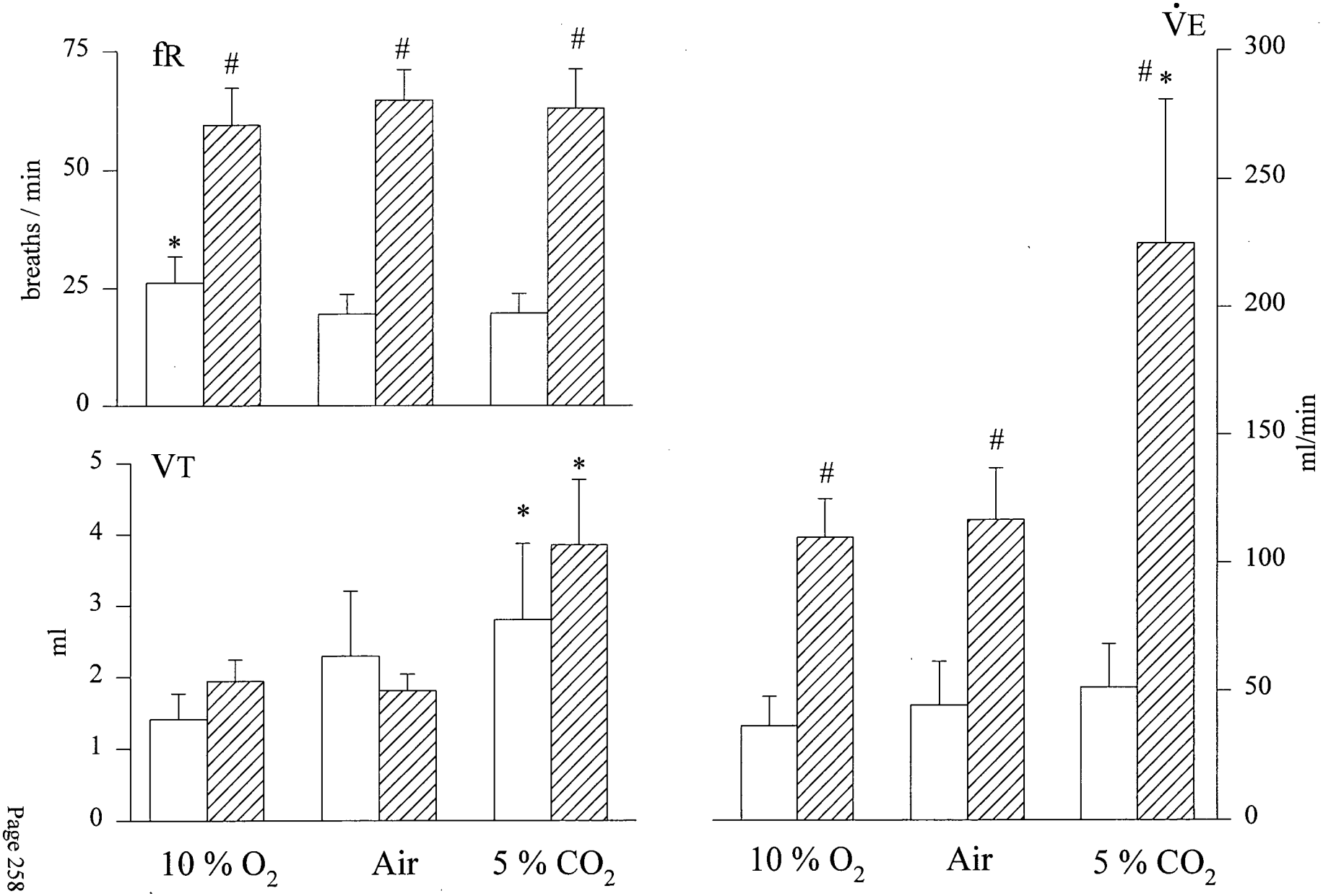

5 % CO2
10 % O2
Air
Volume (VT)
Time (seconds)
0
1
2
3
4
5

Fig. 8.9

Mean (± standard error) values for breathing frequency (f_R) tidal volume (V_T) and ventilation (V_E) in vagotomized squirrels, during exposure to air, 10% O_2 and 5% CO_2, after either bilateral microinjection of MK-801 into the region of the medial and lateral Parabrachial and Kölliker-Fuse Nuclei (open bars), or intravenous administration of MK-801 (hatched bars). The "*" denotes a significant difference from treatment values during exposure to air, while the "#" denotes a significant difference from the microinjection values during exposure to that gas ($P<0.05$).


fR
breaths / min
75
50
25
0
VT
ml
5
4
3
2
1
0
$\dot{V}E$
ml/min
300
250
200
150
100
50
0
10 % O_2
Air
5 % CO_2

The conclusion of this earlier study was that either the hypoxic ventilatory response required the activation of NMDA receptor-mediated processes, or that the ventilatory changes associated with systemic MK-801 treatment precluded the expression of this response. Both still remain possibilities but must be restricted to the influences of MK-801 outside the PBC.

Despite the presence of an hypoxic ventilatory response, prolonged exposure to hypoxia resulted in the occurrence of an epileptiform EEG which returned to normal following a return to breathing O_2 supplemented air. These results suggest that the ventilatory adjustments which did occur were not sufficient to meet the ventilatory requirements. During the present investigation and those outlined in chapters 3 and 4, vagotomy alone was observed to depress ventilation and induce an epileptiform EEG. It is not surprising, therefore, that the apneustic pattern observed in the present study does not produce adequate gas exchange, nor that the hypoxic ventilatory response during apneustic breathing is not as robust as that normally exhibited by this species.

8.4.5 Apneustic breathing pattern: Hypercapnia

The apneusis observed during hypercapnia was also characterized by a pause (TIP) which occurred between TIA and TEA and a slight reversal of flow during TIP (Fig. 8.4, 6). The magnitude of this flow reversal appeared to be greater than that which occurred during exposure to air. If this flow reversal was due to decreases in inspiratory muscle activation during TIP, or failure of the inspiratory muscles to maintain VT during prolonged activation, then this difference might be expected as VT is increased during hypercapnia and, thus, the forces promoting elastic recoil of the chest would be greater than during exposure to air.

Exposure to hypercapnia, during apneusis, produced an increase in VT mediated solely

by an increase in mean ventilatory flow rate with no change in T_{IA}. This response is similar to that normally exhibited by this species, and typical of hypercapnic ventilatory responses in general (Bradley et al. 1974; Cunningham et al. 1986 q.v.; chapter 3). Thus, these results indicate that a ventilatory response to hypercapnia is present in this species following vagotomy and NMDA receptor antagonism, despite the apneustic breathing pattern. The hypercapnic ventilatory response observed under these conditions is also similar to that observed during the apneustic breathing pattern induced by vagotomy and systemic application of MK-801 (Fig. 8.8, 9). Alterations in tidal volume in response to hypercapnia are, therefore, not dependent on NMDA receptor-mediated processes within or outside the PBC. In the absence of such processes, mechanisms which regulate V_T remain functional.

Both hypercapnia and hypoxia stimulated breathing, yet, neither treatment significantly altered the duration of the end-inspiratory pause. This indicates that inspiratory timing is not responsive to chemoreceptor inputs during apneusis although other aspects of the respiratory cycle are. Although breathing pattern was altered, neither hypercapnia nor hypoxia resulted in significant increases in ventilation. This indicates that the ventilatory changes associated with apneusis were disruptive to ventilation and prevented the precise matching of ventilation and ventilatory demand, despite the changes in V_T and f_R which occurred in response to ventilatory stimuli.

8.5. Conclusion

Antagonism of NMDA-type glutamate receptors within the PBC of vagotomized anesthetized spontaneously breathing ground squirrels results in apneusis, primarily characterized by the development of a pause following inspiration during which ventilatory airflow does not occur. This pause results from the prolonged activation of inspiratory muscles during an extension of the inspiratory phase of the respiratory cycle. The end-inspiratory pause is not influenced by hypoxic or hypercapnic ventilatory stimulation. Both hypoxia and hypercapnia alter breathing pattern in ways normally associated with hypoxic and hypercapnic ventilatory responses in this species, despite the apneustic breathing pattern.

Chapter 9

General Discussion

9. General Discussion

9.1. Anesthetized animals

The ventilatory cycle can be divided into a number of distinct phases based on patterns of ventilatory flow. The first basic division of the ventilatory cycle is into periods of inspiration (T_I) and expiration (T_E). When measuring flow, each or these two divisions can be classified into periods during which flow occurs (T_{IA} and T_{EA}) and periods during which it does not (T_{IP} and T_{EP}) (Fig. 2.1). The latter two periods do not occur under all conditions. Figure 9.1 illustrates the various portions of the ventilatory cycle and how they change during the different experimental treatments.

9.1.1 Vagal feedback

Vagotomy altered breathing pattern by decreasing breathing frequency while increasing tidal volume. Each breath was lengthened via increases in the durations of inspiration (T_I) and expiration (T_E). Thus, vagal feedback in anesthetized ground squirrels normally shortens the period of inspiration, limiting tidal volume, and shortens the period of expiration, enhancing breathing frequency.

Vagal feedback provides both phasic and tonic inputs to respiratory centers. Phasic activity codes for the timing and volume of each lung inflation and is superimposed on tonic vagal activity which codes information regarding inflation state of the lung at the end of the breath. Phasic vagal feedback influences the duration of both inspiration and expiration in different ways during different phases of the ventilatory cycle. Inspiration is stimulated and then inhibited by phasic activity delivered during the middle and the last third of the inspiration, respectively, while phasic input during early- and mid-expiration stimulates

expiration (Feldman and Gautier 1976; Cross *et al.* 1980). Decreases in tonic vagal feedback generally result in a decrease in T_E which decreases the ratio of T_I/T_E, promoting lung inflation and increasing f_R, while increases in tonic feedback are associated with increases in T_E which decrease the ratio of T_I/T_E, promoting deflation (Knox 1973; Trippenbach et al. 1985). Vagotomy results in the loss of both tonic and phasic vagal feedback and is generally associated with a slowing and deepening of breathing. The increase in tidal volume is attributed to an increase in the duration of inspiration resulting from the loss of phasic feedback. The slowing of breathing frequency is due to increases in the duration of both inspiration and expiration. The increase in the duration of expiration would not be expected from a decrease in either tonic or phasic vagal feedback and has been difficult to reconcile (Phillipson, 1974; Sullivan et al. 1978; DiMarco et al. 1981; Trippenbach et al. 1985; Fedorko et al. 1988; Coleridge and Coleridge, 1986 q.v.). The results of the work presented in this thesis are consistent with these previous studies. The increases in T_E, in the present study following vagotomy are particularly large, highlighting the presence of a vagal feedback mechanism that normally shortens T_E and elevates f_R.

9.1.2. NMDA receptor-mediated processes

Systemic application of the NMDA antagonist MK-801 increased breathing frequency. The increase in breathing frequency resulted from a decrease in the duration of inspiration, while inspiration occurred at the same mean inspiratory flow rate, resulting in a slight decrease in tidal volume. Expiratory airflow still occurred over the same time period, but the end-expiratory pause was also shortened. The net result was a large increase in breathing frequency. This is very different from results obtained in other mammals.

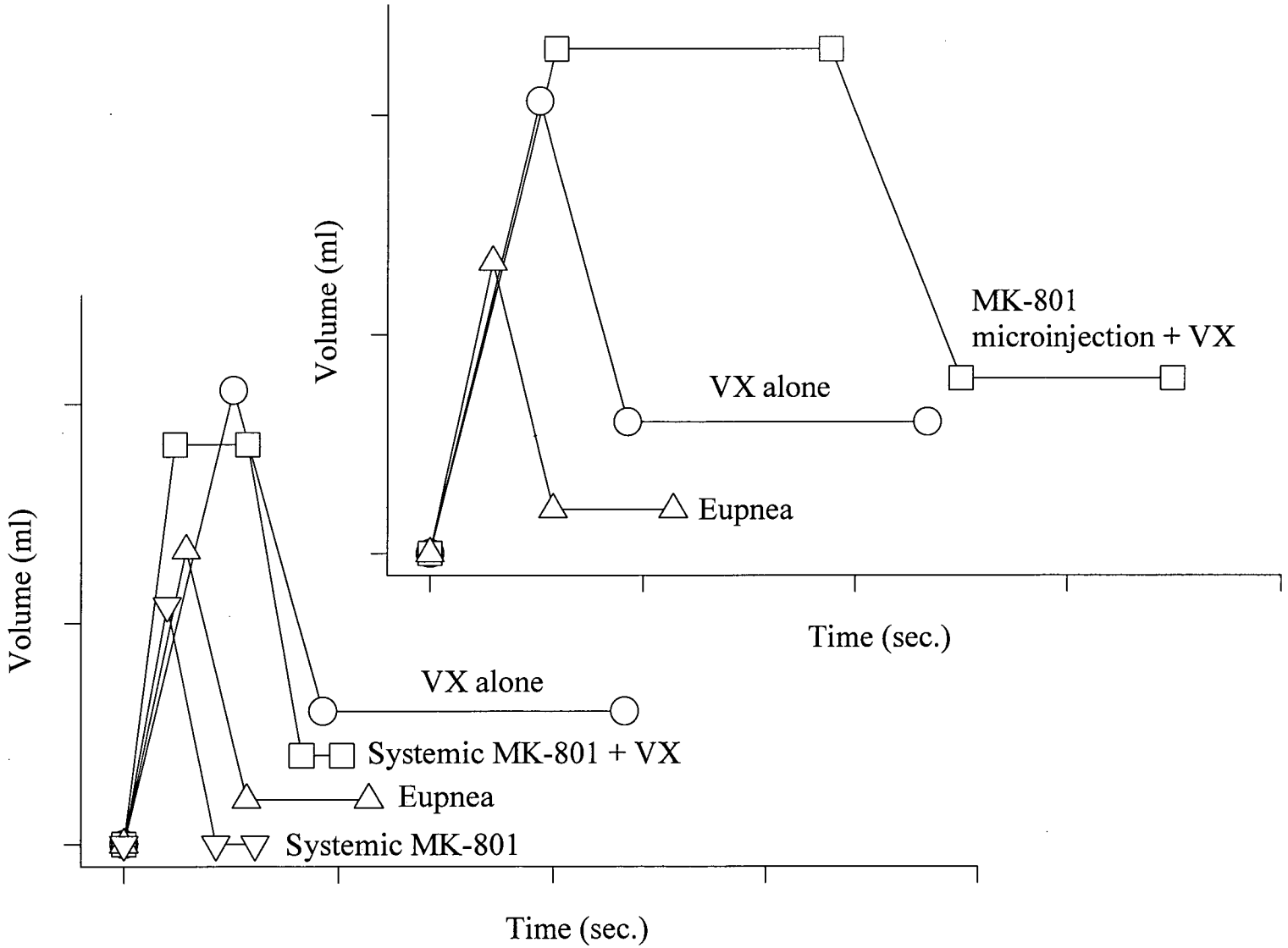
Electrical stimulation within the PBC has been shown to terminate inspiration through an early activation of the inspiratory off-switch (Von Euler and Trippenbach 1975), while lesions in this area delay inspiratory termination (Knox and King 1976). Foutz et al. (1988) have shown that systemic application of MK-801 leads to an increase in T_I in cats, and Fung et al. (1994) have shown that unilateral injection of MK-801 into the PBC in vagotomized rats produced an increase in T_I. These data suggest that it is an NMDA receptor-mediated process in the PBC that activates the inspiratory off-switch. Changes in T_I were normally balanced by reciprocal changes in T_E in these studies which tended to prevent changes in the duration of the ventilatory cycle and hence, in breathing frequency. Thus, MK-801 blockade of the PBC alone in other studies either did not change or decreased breathing frequency.

In the present study, the influences of systemic MK-801 treatment on T_I were opposite to those observed in other studies. This difference could represent a species-specific difference between squirrels and other types of animals tested. This difference could also result from the interruption of NMDA receptor-mediated processes outside the PBC. Systemic administration of MK-801 will block NMDA receptor-mediated processes throughout the central nervous system. The influences of MK-801 administration into the PBC alone were not assessed in the present study. The possible influences of NMDA receptors within and outside the PBC may be deduced by considering the results of systemic MK-801 treatment alone and the differences between systemic MK-801 and MK-801 microinjection into the PBC following vagotomy (Fig. 9.1).

Systemic administration of MK-801 results in a decrease in the durations of inspiration and expiration. Vagotomy alone results in an increase in the durations of inspiration and expiration. Systemic application of MK-801 in conjunction with vagotomy results in apneusis

Figure 9.1

A representation of mean inspiratory volumes, durations inspiratory and expiratory flow, and the pauses occurring at the end of inspiration and expiration of anesthetized animals following various treatments (as labeled). The left panel illustrates data from chapter 7 employing systemic administration of MK-801 while the right panel illustrates data from chapter 8 employing microinjection of MK-801 into the pneumotaxic center. Note that in different plots the end-expiratory volumes have been offset from zero for clarity.


Volume (ml)
MK-801
microinjection + VX
VX alone
Eupnea
Time (sec.)
Volume (ml)
VX alone
Systemic MK-801 + VX
Eupnea
Systemic MK-801
Time (sec.)

but still decreases the duration of expiration from that observed following vagotomy. Microinjection of MK-801 into the PBC following vagotomy also results in apneusis, but during this apneusis the duration of inspiration is considerably greater than that observed following vagotomy alone, while the duration of expiration is not different from that following vagotomy alone. These combined results indicate that NMDA receptor-mediated processes within the PBC normally limit the duration of inspiration and that NMDA receptor-mediated processes outside the PBC normally extend the duration of inspiration. The lack of change in T_I with systemic application of MK-801 suggest the influence of these processes is about equal. These results also indicate that NMDA receptor-mediated processes within the PBC do not influence the duration of expiration, but that NMDA receptor-mediated processes outside the PBC extend the duration of expiration. If this interpretation is correct, then descending influences from the PBC and vagal feedback would both appear to activate the inspiratory off-switch in the golden-mantled ground squirrel as they do in other species.

9.1.3 Control of different aspects of the ventilatory cycle

This raises the question of the extent to which vagal feedback and descending influences from the PBC are redundant. Based on the interpretation put forward in the previous section, the data from the present investigation indicate that different aspects of the ventilatory cycle are modulated in different ways by vagal feedback and NMDA receptor-mediated processes.

Inspiration : The duration of inspiration is limited by both vagal feedback and NMDA receptor-mediated processes in the PBC. Both inputs appear to have similar influences on T_I.

Expiration: While the duration of expiration is limited by vagal feedback, NMDA receptor-mediated processes within the PBC appear to have no influence on the duration of

expiration.

Inspiratory flow: The mean rate of inspiratory flow is not influenced by either vagal feedback or NMDA receptor-mediated processes within the PBC. This rate remains constant following removal of either of these modulating factors.

The ventilatory cycle: All of these factors influence the total duration of the ventilatory cycle and, hence, breathing frequency. Since vagal feedback limits the duration of the T_I and T_E, while NMDA receptor-mediated processes within the PBC only appear to limit T_I, the results suggest that vagal feedback is more influential in enhancing breathing frequency.

9.1.4. Interaction between vagal and NMDA-receptor mediated processes

In the present investigation, both vagal feedback and NMDA receptor-mediated processes within the PBC appear also to limit the duration of inspiration. One or other of these inputs was required to terminate inspiration and the removal of both resulted in apneusis in the same manner as have been identified in other studies (Von Euler et al 1970; Von Euler and Trippenbach 1975 and 1976; Wang et al. 1993, Fung et al. 1994; Bianchi et al 1995 q.v.).

Systemic MK-801 and Vagotomy: Apneusis also occurred following systemic administration of MK-801 and vagotomy. Inspiration was greatly prolonged. Tidal volume and the duration of inspiration, were greater than during eupnea, yet the duration of the overall ventilatory cycle was less than that observed during eupnea in intact animals. The difference between apneusis resulting from systemic administration of MK-801 and that observed following MK-801 microinjection into the PBC must be due to the removal of NMDA receptor-mediated processes outside the PBC.

Systemic administration of MK-801 alone resulted in a tachypnea, characterized by a

slight increase in inspiratory flow and decreases in the durations of inspiration and the end-expiratory pause. The same alterations in breathing pattern appear to be superimposed upon the apneusis which occurs with systemic MK-801 administration following vagotomy. Thus, although apneusis occurs, mean inspiratory flow rate and the durations of inspiration and expiration are less than those which occur during the apneusis following MK-801 microinjection into the PBC.

The differences between these two examples of apneusis are significant. It is generally assumed that the only NMDA receptor-mediated processes which are involved in the modulation of breathing pattern are located within the PBC, and that NMDA receptor-mediated processes outside the PBC are not involved in the modulation of respiratory pattern (Ling et al. 1994; Cassus-Solanis et al. 1995). Thus, MK-801 is commonly administered systemically to assess the involvement of NMDA receptors located within the PBC. Data from the present investigation demonstrate that, in ground squirrels, this basic assumption does not hold.

Apneusis does not represent the intrinsic output of the medullary CRG. Apneusis is reduced when PBC lesions or transections which remove the PBC are followed by more caudal transections or lesions in the caudal pontine reticular formation, and is completely eliminated following pontomedullary transection (Wang et al. 1957; Feldman 1986; Wang et al 1993; St.John 1996; Bianchi et al. 1995 q.v.). In some studies, the breathing pattern that remains appears eupneic while in others it consists of gasping (Wang et al. 1957, Smith et al. 1990; St.John 1996). While the reasons for these differences remain controversial all results indicate that the apneusis is supported by "processes" which are located in the caudal pons. Lumsden (1923) originally postulated that an "apneustic center" was located in this area. Despite extensive investigation, such a center has not been identified (St.John 1996). Whatever these

processes may be, they appear to be the substrate upon which vagal feedback and descending inputs from the PBC act.

9.1.5 Non-NMDA receptor mediated processes in the PBC

The influences of non-NMDA receptor-mediated processes in the PBC were not assessed in the present investigation. In both rats and cats, functional blockade of NMDA receptors in the PBC has the same influence as PBC lesion, while antagonism of non-NMDA receptors has no effect (Fung et al. 1994; Ling et al. 1994; Pierrefiche et al. 1994; Fung et al. 1994; Bonham 1995 q.v.). The PBC does receive both NMDA and non-NMDA receptor-mediated inputs, however (Jhamandas and Harris 1992). It is possible that non-NMDA type processes mediate some aspects of pneumotaxic center function. The focus of the microinjection portion of the study was to confirm that the apneusis observed following systemic MK-801 treatment and vagotomy was due to NMDA receptor-mediated processes located within the PBC, rather than to characterize the role of excitatory amino acids in the PBC. Such a characterization was beyond the scope of the present investigation.

9.1.6. Vagal and PBC inputs and the expression of normal chemosensitivity

A) Hypercapnia:

Intact animals: Chemoreceptor-mediated input can modulate breathing pattern by altering breathing frequency and tidal volume in ground squirrels. Hypercapnia, primarily lead to an increase in tidal volume through increases in mean inspiratory flow rate rather than through increases in inspiratory duration. This suggests that hypercapnia somehow altered the number of inspiratory motor units activated by the CRG during inspiration, but did not alter the

rhythm itself. The response seen in the present study was similar to that normally exhibited by this species, and typical of hypercapnic ventilatory responses in general (Bradley et al. 1974; Cunningham et al. 1986; chapter 3). Vagotomy itself resulted in a decrease in breathing frequency and an increase in V_T. Following vagotomy, the hypercapnic ventilatory response, which was primarily an increase in V_T, was greatly reduced. The elevations in V_T due to vagotomy appeared to preclude V_T increasing much further.

During the apneusis following microinjection of MK-801 into the PBC in vagotomized animals, hypercapnia still produced small increases in tidal volume but the same constraints still appeared to apply. Although the magnitude of this response was diminished during apneusis, these data indicated that a significant hypercapnic response could occur during this breathing pattern.

B) Hypoxia:

Intact animals: Hypoxia stimulated f_R by shortening the durations of inspiration and expiration. These changes resulted in a decrease in the duration of the ventilatory cycle, suggesting that hypoxia somehow influenced the CRG itself. The durations of inspiration and expiration did not shorten to the same degree. There was a greater decrease in expiration, thus, as the ventilatory cycle decreased, the duty cycle (T_I/T_{TOT}) increased. The hypoxic ventilatory response was not greatly altered by vagotomy. During the apneusis following microinjection of MK-801 into the PBC in vagotomized animals, however, the hypoxic ventilatory response was reduced but still present. This indicated that NMDA receptor-mediated processes occurring within the PBC were not required for the full expression of this response. These results agree with previous studies which indicate that, although lesions within the PBC alter some aspects of

ventilatory chemoresponses, these effects not occur following antagonism of NMDA receptors in the PBC (St.John, 1977; Fung et al. 1994).

9.1.7. Applicability of conclusions

The influences of vagotomy and MK-801 treatment reported in the present investigation are generally in agreement with other such studies. The tachypnea associated with systemic MK-801 treatment is not otherwise generally observed. Data from studies on unanesthetized animals strongly suggest that this may reflect specific influences of urethane anesthesia and this is discussed in the next section.

9.2. Unanesthetized Animals

9.2.1. Role of the NMDA receptor mediated processes

Treatment with MK-801 had no significant influence on breathing pattern in unanesthetized animals. This treatment no longer produced the tachypnea it did during anesthesia. This indicated that there was no significant NMDA receptor-mediated inhibition of breathing in awake animals which could have been reversed by functional blockade of NMDA receptors. This does suggest, however, that the depression of breathing frequency which occurred between wakefulness and anesthesia was due to the activation of an NMDA receptor-mediated process

9.2.2. Role of vagal feedback.

In unanesthetized animals, vagal blockade abolished spontaneous ventilation. Breathing stopped on expiration and the apnea was centrally mediated rather than obstructive. This apnea

represented a suppression of ventilatory drive that could not be overcome by the chemoreceptor input associated with the changes in blood-gas which must have occurred during the initial stages of apnea. These data indicate that the vagal feedback which was shown to stimulate breathing in anesthetized animals, must normally have overcome an additional profound ventilatory inhibition in unanesthetized animals. The source of this inhibition is unknown but must have been removed by anesthesia.

Clearly PBC input alone could not overcome this inhibition and the source of the ventilatory inhibition which resulted in the centrally mediated apnea was not reliant on an NMDA receptor-mediated process. Since the general depression of f_R associated with anesthesia was removed by MK-801 treatment, indicating that this depression was dependent on an NMDA receptor-mediated process, there must be two distinct sources capable of inhibiting ventilation. The first is a non-NMDA receptor-mediated process which prevents spontaneous ventilation in unanesthetized animals and is overcome by vagal feedback. The magnitude of this is greatly diminished during anesthesia, although it still may be present in a reduced form as vagotomy still results in a depression of f_R during anesthesia. As vagotomy is generally associated with a slowing and deepening of breathing, this non-NMDA receptor-mediated source of ventilatory inhibition is likely to be common amongst species. The magnitude of this ventilatory inhibition would appear to be highly variable, as removal of vagal feedback in adults of other species does not result in the catastrophic ventilatory depression observed in squirrels. It has been observed in neonatal rats (Fedorko et al. 1988), and the implications of this have been discussed in chapter 3.

The second is an NMDA receptor-mediated depression of f_R which is produced by urethane anesthesia, and which is overcome by MK-801 treatment. Different species

anesthetized with different agents do not generally exhibit a tachypnea following MK-801 treatment. Thus, the NMDA receptor-mediated ventilatory depression may be specifically associated with the pharmocological influences of urethane, or may be specific to ground squirrels. There is preliminary evidence indicating that Sprague-Dawley rats also exhibit a tachypnea following MK-801 treatment during urethane anesthesia (Boon and Milsom, unpublished), suggesting that the ventilatory depression is specific to urethane anesthesia rather than the squirrels *per se.* Which aspect of urethane anesthesia causes this depression is, however, unknown.

9.2.3. Intrinsic properties of the CRG

For such a profound ventilatory depression to occur, the CRG must either be tonically suppressed by an inhibitory input requiring vagal input to over-ride this inhibition, or be active below a threshold requiring activation from vagal input to bring its intrinsic activity above this threshold. Vagal feedback is less important to the expression of CRG activity during anesthesia and hibernation, as ventilation persists following vagotomy under these conditions. Anesthesia and hibernation are both generally associated with a reduction in neural function. Thus, the reduced importance of vagal feedback under these conditions suggests that an inhibitory input suppresses ventilation in unanesthetized animals and that the magnitude of this inhibition is reduced during anesthesia and hibernation.

9.3. Hibernating animals

9.3.1. Vagal feedback

Vagal input in anesthetized euthermic squirrels stimulates breathing by facilitating

breathing frequency. Vagal blockade in both euthermic and hibernating squirrels resulted in significant decreases in ventilation. Following vagal blockade, hibernating animals produced breathing episodes as frequently but took fewer breaths in each episode, while the pattern of individual breaths and the instantaneous frequency of breaths in each episode were not changed. Vagal input alone in hibernating animals, however, continues to stimulates breathing by facilitating overall breathing frequency. The role of vagal feedback appears to be different during hibernation than during euthermia. Tidal volume and the durations of both inspiration and the ventilatory cycle no longer appear to be under the influence of vagal feedback. These variables were regulated by phasic vagal information in euthermic animals, suggesting that phasic vagal activity is not involved in ventilatory pattern control during hibernation. During hibernation, however, some aspect of vagal feedback facilitates the number of breaths occurring in each episode rather than the breaths themselves.

Vagotomy did not result in ventilatory arrest during hibernation as it did in unanesthetized euthermic squirrels. This suggests that hibernating animals do not require vagal feedback to overcome a profound ventilatory depression as was the case in euthermic unanesthetized animals. Hibernation itself must remove the profound ventilatory depression which was active in euthermia so that spontaneous ventilation persists following vagotomy. In this respect, hibernation is similar to anesthesia. During anesthesia, however, some ventilatory depression persisted and tended to prolong the end-expiratory pause and be opposed by vagal feedback. During hibernation the ventilatory depression appears to be totally absent as the pause between breaths and instantaneous breathing frequency were unaffected by vagal blockade.

9.3.2. NMDA receptor-mediated processes

During hibernation, breathing occurs in episodes. MK-801 treatment reduces the pause between breaths within an episode (TEP) resulting in an increase in the instantaneous frequency of breathing in each episode. The lengths of the episodes do not change, but even so, each episode contains a greater number of breaths. There was a non-significant increase in the length of the apnea which occurred between each breathing episode associated with this treatment and, thus, overall breathing frequency and ventilation were unchanged. It is not known whether this occurs because of the influences of NMDA receptor antagonism within or outside the PBC, yet this result appears similar to that which occurred in euthermic anesthetized animals. In both cases, general application of MK-801 resulted in a decrease in the duration of the end-expiratory pause and an increase in instantaneous breathing frequency. The degree to which breathing frequency changed was similar in both euthermic anesthetized and hibernating squirrels.

9.3.3 Hibernation and the CRG

The only change in the pattern of individual breaths observed during hibernation is the decrease in the pause between breaths within an episode and the resulting increase in instantaneous fR occurring after MK-801 treatment. Otherwise, TI, TEA TEP and VT were all fixed during hibernation. These data suggest that breathing during the episodes represents the intrinsic properties of the CRG modulated only by an NMDA receptor-mediated process which tonically inhibited the cycle time of the CRG by prolonging the end-expiratory pause following expiration.

Hypercapnia and vagal feedback influence breathing during hibernation, yet their

influence occurs at the level of the episode and not within the cycle of individual breaths. These factors do not influence the cycle time of the CRG *per se*, but rather they determine the period over which this cycle is expressed. Both hypercapnia and vagal feedback stimulate ventilation by prolonging the breathing episode.

9.3.4 Interaction between vagal and NMDA-receptor mediated processes

NMDA receptor-mediated processes inhibit the number of breaths in each episode by inhibiting the cycle time of the CRG and, thus, limiting the number of cycles which can occur over the set duration of the episode. Vagal feedback stimulates the number of breaths in an episode by prolonging the episode and the period over which the CRG can be active. NMDA receptor-mediated processes and vagal feedback appear to act in opposition but they are acting on different aspects of breathing pattern. Beyond this, however, both of these processes must facilitate the episode itself as the episode persists in the absence of either one alone, while in the absence of both breathing episodes no longer occur.

Following the removal of NMDA receptor-mediated processes and vagal feedback breathing occurs as a pattern of relatively evenly spaced individual breaths. Breathing episodes no longer spontaneously occur, nor can they be triggered by hypercapnia which previously increased the number of breaths in each episode. The factors responsible for clustering breaths into episodes has, thus, been removed. These data indicate that the episodes are supported through interaction between NMDA receptor-mediated processes and vagally-mediated processes in the same manner as eupnea is supported in euthermic anesthetized animals.

Apneusis does not occur during hibernation. The reason for this is enigmatic. Apneusis occurs in euthermic animals because the inspiratory off-switch mechanism is not triggered by

either PBC-mediated or vagal-mediated mechanisms and, as such, inspiration persists until the inspiratory cycle is terminated by an additional and as yet undefined mechanism. The latency of this tertiary inspiratory-termination mechanism which is not mediated by vagal feedback or NMDA receptors in the PBC, and the expiratory duration which follow, define the cycle duration for the apneustic breath. It may be that PBC-mediated and vagally-mediated mechanisms do not trigger the inspiratory off-switch mechanism during hibernation and that inspiration is normally terminated by this tertiary inspiratory-termination mechanism. Inspiratory duration during hibernation is normally two to three times greater than that of euthermia and similar in length to the duration of apneusis in euthermia. Thermal constraints on pulmonary mechanics and on muscle and nerve function may dictate that inspiration requires the full period of activation to achieve the specified tidal volume. Thus, as PBC-mediated and vagally-mediated mechanisms do not influence inspiratory duration and are not involved in triggering the end of inspiration, their removal does not result in apneusis, rather, the normal breath during hibernation may already be an apneusis.

9.3.5. Influences of temperature alone

Both PBC-mediated and vagal-mediated inspiratory-termination mechanisms should be proportionately deactivated by the decrease in body temperature associated with hibernation. As inspiratory termination mechanisms became less effective, the resulting breath would become prolonged and more closely represent the activity of the intrinsic "apneustic mechanism" or of inspiratory termination mechanisms of the CRG itself. It would be expected that these latter mechanisms would be similarly influenced by temperature and is intriguing, therefore, that the inspiratory duration and ventilatory cycle duration of breaths during

hibernation were approximately equal to those observed during apneusis in euthermic animals. This suggests that the decrease in temperature does not reduce the total ventilatory cycle duration and thus, that operation of the CRG is somehow temperature independent.

Kilduff et al. (1988 and 1990) have speculated that squirrels selectively preserve the metabolic activity of specific brainstem nuclei from the general depression associated with low body temperatures during hibernation. Thus, it is possible that there are processes at work during hibernation above and beyond the passive influences of temperature alone. The specific nature of these processes have yet to be determined.

9.4 Assessment of initial hypotheses

The present investigation was designed to address a series of questions, concerning NMDA receptor-mediated processes within the pneumotaxic center and vagally- mediated processes in the control of breathing in ground squirrels. Generally, these processes had been shown to interact and modulate breathing pattern in mammals. It had been suggested, however, that this generality may not be true in all rodents. The first portion of this study assessed the importance of these processes and their interaction in ground squirrels. The results indicated that, for the most part, these processes were involved in the control of breathing in squirrels to the same degree as other mammals.

The second portion of the study was concerned with the generation of episodic breathing patterns during hibernation. There was evidence to suggest that breathing episodes were produced by influences stemming from the higher brainstem. This study, therefore assessed the importance of NMDA receptor-mediated and vagally- mediated processes on breathing pattern in hibernation. The results indicate that the influences of these processes

persist during hibernation and suggest that interaction between the two is responsible for the genesis of breathing episodes.

The first series of experiments investigated the specific hypothesis that vagal afferent feedback influences the resting breathing pattern of spontaneously breathing golden mantled ground squirrels during unanesthetized sleep and wakefulness, anesthesia and natural hibernation. The results supported this hypothesis and indicated that vagal feedback was essential for the expression of spontaneous breathing in unanesthetized squirrels, that it determined inspiratory and expiratory durations in anesthetized squirrels, and that it determined the duration of breathing episodes but not the characteristics of individual breaths during hibernation.

These experiments also assessed whether vagal feedback influenced the ventilatory responses to chemoreceptor stimuli which occur in anesthetized, euthermic and hibernating squirrels. It was determined that ventilatory responses persisted following removal of vagal feedback, although the changes in breathing pattern associated with this treatment did interfere with these responses.

The second series of experiments determined whether NMDA receptor-mediated processes influence the resting breathing pattern of ground squirrels during unanesthetized sleep and wakefulness, anesthesia and natural hibernation. These results indicated that NMDA receptor-mediated processes were essential for the expression of sleep or sleep-like states of anesthesia; that they did not generally influence breathing in unanesthetized animals yet were responsible for the depression of breathing frequency during anesthesia; and depressed breathing within episodes during hibernation, they were not responsible for the production of breathing episodes themselves.

These experiments, too, assessed whether NMDA receptor-mediated processes influenced the ventilatory responses to chemoreceptor stimuli in unanesthetized, anesthetized and hibernating squirrels. The results indicated that NMDA receptor-mediated processes were responsible for the ventilatory response to hypoxia, but not the response to hypercapnia.

A third series of experiments assessed the hypothesis that vagal feedback and NMDA receptor-mediated processes in the pons interact to prevent apneusis and together determine the resting breathing pattern of euthermic ground squirrels. These studies determined that NMDA receptor antagonism did not prevent the ventilatory arrest following vagotomy in unanesthetized animals, yet that interaction between these factors did balance breathing pattern and prevent apneusis in anesthetized squirrels. These studies also indicated that NMDA receptor-mediated processes located outside the pneumotaxic center had significant influences on breathing pattern and were responsible for the interruption of the hypoxic ventilatory response.

These studies also assessed whether vagal feedback and NMDA receptor-mediated processes in the pons interact to produce breathing episodes in hibernating squirrels. The results indicated that this interaction was responsible for clustering breaths into episodes.

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