The influence of NMDA receptor-mediated processes on breathing pattern in ground squirrels

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Abstract

The effects of blockade of N-methyl-D-aspartate (NMDA) type glutamate receptors by a non-competitive antagonist (MK-801) on cortical arousal, breathing pattern and ventilatory responses to hypoxia (10% O2 in N2) and hypercapnia (5% CO2 in air) were assessed in anesthetized (urethane) and unanesthetized golden-mantled ground squirrels (Spermophilus lateralis). Intra-cerebroventricular administration of MK-801 did not alter ventilation during wakefulness, although it did alter the pattern (breathing frequency and tidal volume components) of the hypercapnic ventilatory response, and suppressed the ventilatory response to hypoxia. Animals did not sleep following treatment with MK-801, and intravenous administration of MK-801 prevented expression of the sleep-like state normally observed in anesthetized animals. In anesthetized animals MK-801 elevated breathing frequency to levels observed without anesthesia, and suppressed the hypoxic ventilatory response. These data suggest that NMDA-type glutamatergic receptor-mediated processes influence cortical arousal and facilitate depression of breathing frequency during anesthesia and the hypoxic ventilatory response. Such processes are not essential for the hypercapnic ventilatory response. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

It has long been recognized that inputs from the region of the pons consisting of the medial and lateral Parabrachial and Kölliker-Fuse nuclei (the Parabrachial complex, PbC) modulate breathing pattern (see Bianchi et al. (1995) for review). More recently, 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 (hereafter referred to as NMDA receptors), and that this receptor type is otherwise relatively scarce in midbrain and brainstem respiratory regions. Consistent with this, while it has been demonstrated that those functions of the PbC associated with ventilation are mediated through NMDA receptors, the rhythmonic properties of the brainstem and reflex ventilatory responses, such as the Breuer–Hering inspiratory-termination reflex, do not rely on NMDA receptor-mediated processes (Karius et al., 1991; Funk et al., 1993; Pierrefiche et al., 1994; Bonham, 1995). Disruption of NMDA receptors with specific antagonists has, thus, been used to investigate the role of the PbC in the production of breathing pattern (Connelly et al., 1992; Ling et al., 1993; Fung et al., 1994; Bonham, 1995; Cassus-Soulanis et al., 1995).

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. While it has been suggested that the rodent respiratory control network is identical to that of the cat (Wang et al., 1993; Fung et al., 1994), rodents have not reacted as uniformly to antagonism of NMDA receptors as have cats (Montreau et al., 1990; Connelly et al., 1992; Cassus-Soulanis et al., 1995; St.John, 1996). Conflicting reports have brought 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 variability between species as well as between strains of rodents in this regard.

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 such as sleep and wakefulness, and contributes to the effects that such states have on ventilation (Phillipson and Bowes, 1986; Bianchi et al., 1995). As such, NMDA receptor blockade may have a secondary effect on ventilation stemming from a primary effect on central activation state. This potentially confounding factor is rarely taken into account as few studies measure patterns of cortical activity or consider central activation state.

During anesthesia, at least with urethane, the golden-mantled ground squirrel exhibits distinct ‘states’ of central activation characterized by patterns of cortical activity which resemble either unanesthetized wakefulness or slow-wave sleep (Grahn and Heller, 1989). These alterations in state have significant effects on breathing, and the alterations in breathing associated with transitions between ‘wake-like’ (State I) and ‘slow-wave-sleep-like’ (State III) states are similar to those which occur as unanesthetized squirrels cycle between wakefulness and sleep (Hunter and Milsom, 1998). Thus, as in the studies of unanesthetized animals, NMDA receptor blockade in anesthetized animals may influence breathing by changing the cortical activation state, and may also influence the effects of anesthesia itself.

Given this, the present investigation was designed to assess the influences of NMDA receptor blockade on cortical activation, the pattern of spontaneous breathing and ventilatory responses to hypoxia and hypercapnia in golden-mantled ground squirrels. In this study care was taken to account for the possible indirect effects of blockade on cortical activation state as well as to examine the influence of anesthesia on the role of this receptor group in the control of ventilation. Our long term goal is to examine the role of the PbC in producing the dramatic changes in breathing pattern that accompany hibernation in this species. Toward this end, it is imperative that we understand this role in producing the resting breathing pattern in normothermic animals.

Given this, the present study was designed to examine the breathing pattern of ground squirrels, so that we could then demonstrate how this changes in hibernation.

2. Methods

2.1. Animals

Experiments were performed on 13 adult golden mantled ground squirrels (Spermophilus
lateralis, $235 \pm 5$ g) of mixed sex. These animals were originally collected from a wild population by a supplier in Redding, CA, and were maintained in a research colony for at least 1 year prior to experimentation. Squirrels had access to lab chow and water ad lib supplemented intermittently with sunflower seeds and fresh fruit. The animals were held in a controlled environment chamber with a set photoperiod of 12 h light, 12 h dark. The photoperiod was altered during the winter to facilitate hibernation. All studies were carried out in the late spring and summer, at approximately the same time of day.

2.2. Instrumentation

2.2.1. Chronic study

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. 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 Hunter and Milsom (1998). Electrode wires were run subcutaneously to a 10-pin connector which was cemented to the skull with dental epoxy.

The 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 the 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.

2.2.2. Acute study

Squirrels used in this series of experiments were anesthetized with a 5 ml kg$^{-1}$ intra peritoneal injection of a 20% solution of urethane (Sigma, dose = 1 g kg$^{-1}$) in saline, and allowed to stabilize for 1 h. Supplemental anesthesia, to facilitate surgery, was induced using vaporous halothane (Wyeth-Ayerst, 3.5% in air) administered via a tight fitting mask. Four cranial EEG, two ECG and two EMG electrodes were implanted in the skull, rib-cage musculature, and shoulder musculature, respectively. The treachea was cannulated and an incision was made in the pubic region and cannulae were implanted in a femoral artery and vein to allow blood pressure assessment and intravenous infusion of saline and pharmacological agents. Arterial and venous cannulae 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 $36 \pm 1^\circ$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.

2.3. Measurements (acute and chronic studies)

During the chronic study, ventilation was measured in unanesthetized animals using the modified whole body plethysmograph technique of Jacky and coworkers (Jacky, 1978, 1980; McArthur and Milsom, 1991). The plethysmograph consisted of a $10 \times 10 \times 10$ 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 L min\(^{-1}\). 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 22–25°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.

A novel technique was used to calibrate the plethysmograph during each test. With the animal in the apparatus, a known volume (V\(_{CAL}\); 1–1.5 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 pumped into the chamber and was, thus, dependant on both the volume and frequency of the injection. Traditionally, as the height of the pressure peak is taken to indicate the volume of either the calibration injection or breath, a flow-through plethysmograph must be calibrated dynamically to account for the frequency-dependence of this response (Jacky, 1978; Epstein and Epstein, 1978; Epstein et al., 1980; Jacky, 1980; McArthur and Milsom, 1991). Briefly, recommended calibration techniques require that calibration volumes be administered in a range of frequencies which match the frequencies of ventilation to be measured (although it would be more appropriate that such injections be made with durations matching those of inspiration). From these data, relationships between volume and peak pressure can be derived which correspond to each frequency. These frequency-dependent relationships can then be used to calculate the volume of a given breath from the peak pressure it generates when it occurs at a given frequency. In the present calibration, the pressure signal was digitally integrated using data analysis software (Advanced 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. Basing this calibration on A\(_{CAL}\), rather than peak pressure, removes the necessity to dynamically calibrate the system to account for frequency-dependence.

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; Epstein and Epstein, 1978; Epstein et al., 1980; Jacky, 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 Ep-
stein and coworkers (Epstein and Epstein, 1978; Epstein et al., 1980) to account for incomplete cooling of end-tidal gas before subsequent inspiration.

Upward deflections in the plethysmograph tracing represent expansion of inspired air. Expelled air cools during expiration, thus, reducing its volume and producing a tracing which generally declines back to a neutral baseline. Note, however, that the plethysmograph records (Fig. 1) depict sudden dips in pressure that occur at the switch from inspiration to expiration followed by periods of relative negative pressure which dissipate as the tracing gradually returns to baseline. The sudden dips reflect the initiation of active expiration, during which expiratory intercostal and abdominal muscles compress the thorax and produce a positive pressure in the lungs. The generation of this positive pulmonary pressure is accompanied by a proportional negative pressure within the plethysmograph chamber. The subsequent periods of negative pressure which return toward baseline reflect the relaxation of expiratory muscles during expiration. Reasons for such predominant active expiration in this species are discussed in the accompanying manuscript (Harris and Milsom, 2000).

During the acute study, ventilation was monitored in anesthetized animals using a differential pressure transducer (Valedyne) attached to a resistance pneumotachograph 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 (1–2 ml) of air were pumped back and forth through the pneumotachograph via a small-animal ventilation pump (Harvard). The resulting peak 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.

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 (WindaQ, DataQ Instruments) sampling at either 100 or 80 Hz per channel during studies of unanesthetized and anesthetized 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.
2.4. Data analysis

2.4.1. Arousal state

Arousal or activation states in unanesthetized and anesthetized animals were determined through subjective examination of EEG and EMG records using established electrophysiological criteria for determination of sleep and wakefulness (Rechtschaffen et al., 1968). Our past investigations of arousal state in this species (Hunter and Milsom, 1998; Hunter et al., 1998) demonstrate that slow-wave sleep (SWS) and the analogous state of anesthesia are associated with an unmistakable large-amplitude slow (2–3 Hz) component to the cortical EEG waveform. Rapid-eye-movement or paradoxical sleep is both infrequent and readily identifiable, as it occurs between or immediately following periods of SWS and is associated with a low-amplitude, high-frequency (wake-like) EEG waveform accompanied by characteristic instability in breathing pattern and postural muscle atonia. Given the focus of the current study, this characterization of arousal state easily allowed us to restrict our analysis to periods which did not include sleep or sleep-like states of anesthesia. Some secondary analysis of EEG waveforms were achieved by frequency analysis using post-hoc analysis software associated with the computer data acquisition system (Advanced CODAS and WindaQ, DataQ Instruments). This was only used to compare our current recordings to those we have made in the past, and to confirm our subjective assessment of sleep and wakefulness.

2.4.2. Ventilation

Ventilation in unanesthetized animals was measured via a whole body plethysmograph, which records the expansion of air due to warming and humidification on inspiration. Respiratory frequency \( f_R \) and tidal volume \( V_T \) were measured during 60-sec 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 sec period. The timing of inspiration \( T_I \) and expiration \( T_E \) of each breath during this 60 sec period were also determined from plethysmograph traces. Since pauses could occur following either inspiration or expiration, both phases were subdivided into periods during which air flow occurred \( (T_{IA} \text{ and } T_{EA}) \) and the pauses during which no air flow occurred \( (T_{IP} \text{ and } T_{EP}) \), for inspiration and expiration, respectively). Mean inspiratory flow rate was determined as the ratio of \( V_T/T_{IA} \). Measurement of \( f_R, V_T, T_{IA}, T_{IP} \text{ and } T_E \) could be obtained easily from the plethysmograph records. The point at which inspiration began was deemed to be the point where the record first showed a rise from the neutral baseline, indicating expansion of inspired air. The end of \( T_{IA} \) was determined as the peak of the recording, or the point where inspired air had expanded to its maximum volume. Precise identification of the termination of airflow in expiration was problematic, however, and this point was determined as the point where the pressure deflection returned to its neutral baseline. Values reported for \( T_{EA} \) and \( T_{EP} \) are less precise.

Ventilation in anesthetized animals was measured via direct assessment of tracheal airflow. Measurement of \( f_R, V_T, T_{IA}, T_{IP}, T_{EA} \text{ and } T_{EP} \), and calculations of \( V_E \) and mean inspiratory flow rate were made from representative 60-sec traces of stable breathing during states with 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 experimental treatment were assessed by comparing the values obtained using one-way repeated measures analysis of 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 to determine normality and equality of variance. Statistical significance was attributed to differences having a level of significance \( (P \text{ value}) \) of less than 0.05 unless otherwise specified.
2.5. Protocol

2.5.1. Chronic study
The six squirrels selected for this portion of the study 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 ml 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⁻¹, 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. Animal were monitored: (i) for at least 2 h to provide initial control values, (ii) for at least 2 h while the test box was supplied with hypercapnic (5% CO₂ in air) gas, (iii) for at least 2 h while the test box was supplied with hypoxic (7% O₂ in N₂) gas (note that hypercapnic and hypoxic exposures were randomized and separated by a 1 h period of exposure to air), (iv) for at least 1 h during exposure to air following a 2 µl injection of saline (sham treatment) into the third ventricle, (v) for at least 1 h following a 2 µl injection of MK-801 solution, and (vi) again during 2 h periods of randomized exposure to hypercapnic and hypoxic gases separated by exposure to air. Supplemental (2 µl) injections of MK-801 solution were made every hour during this procedure.

2.5.2. Acute study
Each of the seven animals used in this study were supplied with air and monitored: (i) for at least 2 h following instrumentation to provide initial control values, (ii) during random exposure to hypercapnic (5% CO₂ in air) and hypoxic (10% O₂ in nitrogen) gas mixtures for at least 30 min periods separated by 30 min of exposure to air, (iii) for at least 1 h following a 2 µl injection of saline (sham treatment) into the third ventricle, (iv) for at least 1 h following a 0.3 ml intravenous infusion of saline (sham) into the venous catheter, (iv) for 1 h following a 0.2–0.3 ml infusion of a 0.15 mg ml⁻¹ 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 (as per Foutz and Champagnat (1988)) and, (v) again following randomized 30 min exposure to hypoxic and hypercapnic gas mixtures separated by 30 min exposure to air. Additional 0.2–0.3 ml infusions of MK-801 solution were administered every hour.

2.5.3. Additional anesthetized animals
The anesthetized animals just described received MK-801 through intravenous injection, while those used in the chronic studies received MK-801 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 (Cassous-Soulanis et al., 1995). To assess the potential difference that route of drug infusion may have had on the results three of the animals instrumented and used in the chronic portion of this study were subsequently retested using the acute (anesthetized) protocol but with injection of MK-801 into the CSF. These animals were anesthetized with a 5 ml kg⁻¹ 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. Arterial and venous catheters were not implanted in these animals, and EEG, EMG, and EKG were monitored using the previously implanted electrodes.

These animals were monitored: (i) for at least 2 h to provide initial control values, (ii) during random exposure to hypercapnic (5% CO₂ in air) and hypoxic (10% O₂ in nitrogen) gas mixtures for at least 30 min periods separated by 30 min of exposure to air, (iii) for at least 1 h following a 2 µl injection of saline (sham treatment) into the third ventricle, (iv) for at least 1 h 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.
3. Results

3.1. Unanesthetized animals (chronic)

3.1.1. Activation state

Unanesthetized animals exhibited distinctive patterns of EEG and EMG characteristic of different phases of vigilance including quiet wakefulness, and both ‘slow-wave’ and ‘rapid-eye-movement’ sleep. The effects of changes in cortical activation state on breathing in this species have been characterized recently by Hunter and Milsom (1998). In the present investigation, cortical activation state was monitored and, to control for indirect effects associated with fluctuations between these states, ventilation was analyzed only during well established periods of quiet wakefulness. Injections of saline into the cerebrospinal fluid had no apparent influence on cortical activation. Within 10 min of MK-801 treatment, however, all animals entered a period of relatively continuous quiet wakefulness. Following MK-801 treatment, sleep-like states of cortical arousal never occurred.

3.1.2. Ventilation

Before MK-801 treatment, hypoxia produced consistent increases in both respiratory frequency ($f_R$) and tidal volume ($V_T$) which, although not statistically significant, together increased ventilation ($V_E$) 149% (Figs. 1 and 2). There were no changes in the timing components of each breath (Fig. 3). Hypercapnia decreased $f_R$ by 68% and increased $V_T$ by 480%, resulting in a 60% increase in $V_E$ (Figs. 1 and 2). Decreases in $f_R$ resulted from increases in durations of non-ventilatory pauses following expiration ($T_{EP}$) and in the duration of inspiration ($T_I$) while increases in $V_T$ resulted from this increase in $T_I$ with no change in the duration of the period of expiratory flow ($T_{EA}$) or the mean inspiratory flow rate (Fig. 3).
3.1.3. Post-MK-801 treatment (cSF)

Injections of saline into the cerebral-spinal fluid had no influence on breathing. Breathing during the quiet-wake-like state of cortical arousal following MK-801 treatment was also not different from that observed during quiet wakefulness (Figs. 1 and 2), nor were there differences in the timing of individual breaths (Fig. 3). Additional doses of MK-801 did not alter breathing further.

Hypoxia subsequent to MK-801 treatment did not alter breathing (Figs. 1–3). Hypercapnia increased $V_T$ by 83%, resulting in a 56% increase in $V_E$ (Fig. 2). Slight decreases in $f_R$ resulted from increases in $T_{EP}$ while increases in $V_T$ resulted from an increase in $T_i$ with no change in the mean inspiratory flow rate (Fig. 3).

3.2. Anesthetized animals (acute)

3.2.1. Activation state

Under urethane anesthesia, two distinct patterns of EEG were observed. The EEG waveform showed a predominance of either high frequency (> 6 Hz) low amplitude activity (State-I) or, high amplitude low frequency (1–4 Hz) activity (State-III). Like animals in natural sleep and wakefulness, anesthetized animals exhibited alterations in breathing pattern associated with states of central activation and these have been characterized recently (Hunter and Milsom, 1998). In the present investigation breathing pattern was characterized only during State I. Intravenous injections of saline did not alter either the central activation

Fig. 3. Representation of mean (with S.E.) 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.
state or breathing. Within 10 min of MK-801 treatment, however, all animals exhibited a consistent State-I type EEG which did not change for the remainder of the experiment.

3.2.2. Ventilation

During State I before MK-801 treatment, hypoxia increased \( f_R \) by 113% without changing \( V_T \), resulting in a 54% increase in \( V_E \) (Fig. 5). Breathing frequency increased due to a decrease in \( T_EP \) (Fig. 6). Hypercapnia increased \( f_R \) by 68% and \( V_T \) by 160%, resulting in a 333% increase in \( V_E \) (Fig. 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).

3.2.3. Post-MK-801 treatment (intravenous)

Breathing was not altered by intravenous injections of saline. Treatment with MK-801, however, resulted in a dramatic 138% increase in \( f_R \), with no change in \( V_T \), resulting in an 116% increase in overall \( V_E \) (Fig. 5). 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 pre-treatment conditions (Figs. 4–6).

Subsequent to MK-801 treatment, hypoxia did not alter either \( f_R \), \( V_T \), or \( V_E \) from levels observed during exposure to air, and there were no changes in the timing components of each breath (Figs. 4–6). Hypercapnia did not alter \( f_R \) but increased \( V_T \) and \( V_E \) by 156 and 145%, respectively (Figs. 4 and 5). Increases in \( V_T \) resulted from an increase in mean inspiratory flow rate (Fig. 6). Additional doses of MK-801 did not alter breathing further.

3.2.4. Post-MK-801 treatment (csf)

Administration of MK-801 directly into the cerebrospinal fluid, rather than into the peripheral circulation, also resulted in a consistent State-I type EEG, and increased \( f_R \), decreased \( V_T \), and increased \( V_E \). Hypoxia did not alter breathing following CSF application of MK-801, while hypercapnia increased \( f_R \), \( V_T \) and \( V_E \). The low number of replicates precludes valid statistical assessment of these data. Subjectively, however, these results appear similar to those observed following intravenous injections of MK-801 in the other anesthetized animals. Again, additional injections of MK-801 solution did not alter breathing further. The influences of MK-801 treatment appeared to subside after approximately 4 h, but an objective assessment of this recovery was not made.

4. Discussion

4.1. Location and mechanism of MK-801 treatment effects

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 pat-
tern 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 effect on ventilation as general administration of this drug (Jhamandas and Harris, 1992; Ling et al., 1993; Wang et al., 1993; Ling et al., 1994). As general administration of MK-801 will antagonize NMDA receptors throughout the CNS, these investigations suggest that the only NMDA receptors directly involved in the control of resting breathing pattern are located within the PbC (Fung et al. (1994), Ling et al. (1994), Cassus-Soulanis et al. (1995), see Bonham (1995) for review). The distribution of NMDA receptors in the CNS of ground squirrels is, at present, unknown and a detailed analysis of this is currently underway. The present study was initiated under the assumption that the distribution of NMDA receptors and the influences of MK-801 treatment in squirrels are similar to those described in other mammalian species studied to date, i.e. that MK-801 will interrupt NMDA receptor-mediated processes throughout the CNS but that the primary effect on ventilation is through its action on receptors within the PbC. It is important to qualify this assumption, however, and to be mindful of secondary effects resulting from disruption of NMDA receptor-mediated processes outside the PbC which may also influence breathing.

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 function of the NMDA receptor, preventing the activity of glutamatergic excitation that would otherwise occur (Huettner and Bean, 1988; Collingridge and Singer, 1991). The results of MK-801 treatment are, thus, attributed to deactivation or the removal of excitatory influences rather than direct inhibition. MK-801 treatment

![Fig. 5](image-url)  
Fig. 5. Mean (with S.E.) values for breathing frequency ($f_R$), tidal volume ($V_T$) and ventilation ($V_E$) of anesthetized animals 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 that condition (either pre or post MK-801), while the ' # ' denotes a significant difference from the pre-MK-801 value during exposure to that gas ($P < 0.05$).
can deactivate either excitatory or inhibitory neurons and, thus, this treatment can result in either a net inhibition or excitation, i.e. a net negative or positive modulation resulting from the removal of either excitatory or inhibitory influences, respectively.

4.2. Unanesthetized animals (chronic)

In the present study breathing was comparable to that observed in other investigations of this species (Hunter and Milsom 1998; Hunter et al., 1998). Treatment with saline or MK-801 did not alter resting breathing during quiet-wake-like states of cortical activation. It is generally accepted that the timing of individual breaths is regulated by both NMDA receptor-mediated processes in the PbC and by feedback from pulmonary mechanoreceptors. The two have been described as complementary control systems, and it is generally believed that the loss of one can be relatively easily compensated for by the other (Feldman et al., 1990). The observation that removal of NMDA receptor-mediated processes, in otherwise intact animals, results in no changes in ventilation indicates either that the influences of such processes on ventilation are insignificant, or that other mechanisms are easily able to compensate for their absence. There is little constancy in the ventilatory responses to this treatment documented in the literature for other species and preparations. In some $f_R$ increases (Loscher et al., 1991; Cassus-Soulanis et al., 1995; Morin-Surun et al., 1995; Ohtake et al., 1998), in others it decreases (Foutz and Champagnat, 1988), while in others still it is unchanged (Greer et al. (1991), Cassus-Soulanis et al. (1995); Morrison (1996)). Such variability may occur because of slight differences in the compensatory balance between NMDA receptor-mediated processes in the PbC and mechanoreceptor feedback between species or, perhaps, because of secondary effects of NMDA receptor disruption, such as influences on cortical arousal, which influence breathing but which have not been accounted for (Cassus-Soulanis et al., 1995).

Prior to MK-801 treatment, hypoxia increased in $V_E$ through slight increases in both $V_T$ and $f_R$, which agrees with other characterizations of hypoxic ventilatory responses in this (Webb and Milsom, 1994; Hunter et al., 1998) and other species (Hedemark and Kronenberg, 1982; Cragg and Drysdale, 1983; Walker et al., 1985; Ohtake et al., 1998). MK-801 treatment eliminated the tendency for $V_T$ to increase with hypoxia and, thus, eliminated the increase in $V_E$. This is also consistent with observations from other studies (Ang et al., 1992; Mizusawa et al., 1994; Miyawaki et al., 1996; Lin et al., 1996; Ohtake et al., 1998). In these studies, antagonism of NMDA receptors within specific regions of the medulla, notably the nucleus of the solitary tract (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, suggesting stimulation of $V_T$ by hypoxia requires NMDA receptor-mediated processes at this site. The trends in the data of the
present study are consistent with this hypothesis, but the modest responses in \( f_R \) and \( V_T \) that we observed lack significance and make further speculation pointless.

Prior to MK-801 treatment, hypercapnia increased \( V_T \) but decreased \( f_R \), resulting in net increase in \( V_E \). While MK-801 treatment did not alter ventilation during exposure to air or hypoxia it increased \( f_R \) and decreased \( V_T \) during hypercapnia. These influences were offsetting such that \( V_E \) was no different during hypercapnic exposure before and after MK-801 treatment. The hypercapnic response following MK-801 treatment, however, consisted of only a modest increase in \( V_T \).

Ameliorated increase in \( V_T \) associated with both hypoxia and hypercapnia following MK-801 may 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 \( V_T \) associated with exposure to hypoxia, it did not reduce the changes in \( V_T \) which occurred during the hypercapnic ventilatory response. NMDA antagonism in the ventrolateral medulla, however, removes the increase in the amplitude of phrenic activity associated with the hypercapnic response, suggesting that the reductions in \( V_T \) noted in the present investigation may be mediated by NMDA receptor-mediated processes in this area (Dillon et al., 1991; Nattie et al., 1993; Nattie and Li, 1995).

The fall in \( f_R \) 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 \( f_R \) component of the hypercapnic ventilatory response of anesthetized or decerebrate cats (St.John, 1977) but this response was an increases in \( f_R \) and not a decrease as in the present study, and it is not known whether the factors within the PbC responsible for this change were influenced through NMDA or non-NMDA receptor-mediated processes. At present we are unable to account for why \( f_R \) fell during hypercapnia in the present study, and where this NMDA receptor-mediated process originated.

4.3. Anesthetized animals (acute)

During urethane anesthesia, ground squirrels exhibit patterns of cortical activity which resemble unanesthetized states of wakefulness or slow-wave sleep (Grahn and Heller, 1989). Changes in breathing associated with transitions between these states are similar to those occurring as unanesthetized squirrels cycle between wakefulness and sleep (Hunter and Milsom, 1998). This has given rise to the hypothesis 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 hypothesis 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.

While treatment with MK-801 did not alter any aspect of breathing in unanesthetized animals, it induced changes in breathing pattern in anesthetized animals. Similar effects were observed regardless of whether anesthetized animals received MK-801 via the peripheral circulation or via the cerebral–spinal fluid. These treatments increased \( f_R \) and \( V_T \). The increase in \( f_R \) was produced by a decrease in both \( T_i \) and \( T_{EP} \). This suggests that an NMDA receptor-mediated glutamatergic process tonically inhibits breathing frequency and acts to lengthen the duration of inspiration and the interval between breaths. In the absence of this inhibition, the interval between breaths decreases and \( f_R \) increases. This pattern of change is identical to that seen in the unanesthetized squirrels, with one notable difference. Levels of \( f_R \), \( V_T \) and \( V_E \) are all considerably lower in anesthetized animals than in unanesthetized animals, reflecting a significant ventilatory depression induced by anesthesia. NMDA receptor antagonism during anesthesia elevated \( f_R \) to levels similar to those observed in unanesthetized animals. Given that MK-801 blocks otherwise active processes, these results indicate that \( f_R \) is depressed in anesthesia through an active inhibition, which is facilitated by glutamatergic activation of
NMDA receptors $V_T$ is still lower following MK-801 treatment during anesthesia than in unanesthetized animals and $V_E$, 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.

The reduction of $T_I$ associated with MK-801 treatment in the present study is unusual. Although Ohtake et al. (1998) observed a similar reduction in $T_I$ following MK-801 treatment in unanesthetized rats, the PbC contributes to the termination of inspiration (von Euler and Trippenbach, 1975) and disruption of NMDA receptor-mediated processes within this area are generally associated with a prolongation of $T_I$ which is exaggerated in the absence of vagal mechanoreceptor feedback (Wang et al., 1993; Fung et al., 1994; Ling et al., 1994; Cassus-Soulanis et al., 1995). As such, $T_I$ was expected to lengthen in response to MK-801 treatment, or to be unchanged as mechanoreceptor-mediated inspiratory termination mechanisms are intact. The observation that $T_I$ is reduced agree with the findings of Ohtake et al. (1998) and suggests that there are additional NMDA receptor-mediated mechanism which act to prolong $T_I$, perhaps located outside the PbC.

Prior to MK-801 treatment, hypoxia increased $f_R$ and $V_E$. This agrees with other observations in this species during anesthesia (Hunter et al., 1998). During anesthesia, hypoxia produced greater increases in $f_R$ than were observed in unanesthetized squirrels, but had no influence on $V_T$. Following MK-801 treatment, the hypoxic ventilatory response was abolished. 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 animals, as in the unanesthetized squirrels, MK-801 abolished the increase in $V_T$ in response to hypoxia, while the increase in $f_R$ remained. In squirrels, however, changes in $V_T$ associated with hypoxia were already eliminated by the anesthesia and now, following MK-801, the animals failed to exhibit further increases in $f_R$ in response to hypoxia. Ohtake et al., (1998) have recently demonstrated that MK-801 treatment does abolish an increase in $f_R$ normally associated with hypoxia in rats. Thus, the lack of an increase in $f_R$ during hypoxia following MK-801 in the present study 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 during normoxia 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. Thus, MK-801 treatment may well have elevated $f_R$ to a maximum frequency. It is not possible to assess whether it is this, or a disruption of an NMDA receptor-mediated process underlying the hypoxic ventilatory response which is responsible for a lack of a hypoxic ventilatory response following MK-801 treatment.

Prior to MK-801 treatment, hypercapnia increased $V_T$ and $f_R$, which then increased $V_E$. This response is similar to those previously observed in anesthetized ground squirrels (Hunter et al., 1998). MK-801 treatment had no influence on the overall hypercapnic ventilatory response although the increases in $f_R$ observed during this response prior to MK-801 treatment 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 recep-
tor-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.

4.4. Influences of NMDA receptor antagonism on cortical arousal

MK-801 administration abolished the expression of SWS or State III and resulted in a relatively continuous state of QW or State I in unanesthetized and anesthetized animals, respectively. 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). These observations suggest that the expression of SWS and State III, (or the production of an EEG pattern indicative of SWS and State III) are 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 or State I.

Variations in cortical arousal state normally occur and such variations, even during anesthesia, cause changes in ventilation. If not taken into account the artificial homogeneity imposed by MK-801 could alter the interpretation of experimental results. One could speculate that including data obtained during normal periods of SWS or State III would bias the results of comparisons with data obtained following MK-801 treatment where these states do not occur. Only by accounting for the influence of MK-801 on the distribution of cortical arousal states, can the influence of this treatment on ventilation per se be determined.

4.5. Influence of NMDA receptors limited to the PbC

It is common to administer MK-801 systemically and to attribute the ventilatory influences of this treatment to the disruption of NMDA receptor-mediated processes within the PbC. The present study identifies influences of MK-801 treatment on the expression of states of cortical activation which, in turn, influence ventilation. We have also identified other influences which are consistent with NMDA receptor disruption outside the PbC. These results call into question the validity of assuming that the ventilatory influences of systemic application of MK-801 are restricted to the PbC and emphasize the need for appropriate controls and consideration for influences stemming from the disruption of NMDA receptor-mediated processes outside the PbC, including changes in states of cortical activation which occur both with and without anesthesia.

In conclusion, ventilation in unanesthetized squirrels is not influenced by MK-801 treatment, while in anesthetized animals, ventilation is stimulated by this treatment. These data indicate that NMDA receptor-mediated processes alone do not regulate the timing of individual breaths, and suggest that glutamate, acting by NMDA receptors, inhibits breathing during urethane anesthesia. Despite the differences in ventilatory responses to MK-801 treatment the data indicate that under both anesthetized and unanesthetized conditions, NMDA receptor-mediated processes facilitate ventilatory responses to hypoxia but are not required for the expression of hypercapnic ventilatory responses.

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